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BIOLUMINESCENCE

By

E. Newton Harvey, Rubert S. Anderson, John B. Buck, Aurin M. Chase, Henry Eyring, and Frank H. Johnson

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INTRODUCTORY REMARKS: A GENERAL SURVEY OF BIOLUMINESCENCE

By E. Newton Harvey

Osborn Professor of Biology, Princeton University, Princeton, New Jersey

Bioluminescence occupies a somewhat paradoxical position among biological subjects. It is not only a specialized and restricted field of inquiry but at the same time a very broad one. It is restricted in that relatively few of the very large number of animal or plant groups have developed the singular ability to produce light, and broad in that it presents problems of the greatest interest to every branch of biology—to the systematist, histologist, morphologist, physiologist, biochemist, and ecologist as well as to the student of animal behavior and of evolution. Furthermore, the beauty and the mystery of animal light have always aroused the interest of the traveler and the student of other fields such as pure chemistry and physics.

Among the great chemists and physicists of the past who have taken a more than casual interest in bioluminescence are Boyle, Newton, Franklin, Priestly, Réaumur, Dessaignes, Becquerel, Davy, and Faraday. Many naturalists have been fascinated by luminous organisms, and it is rather surprising to find that Darwin, despite the variety of his travels and the breadth of his interest, casually mentions luminous animals and draws no inferences for the theory of natural selection from the widespread ability of many living forms to emit a cold light. Among biologists who have made a special study of animal light may be mentioned the names of Anderson, Beijerinck, Buck, Chase, Dahlgren, Dubois, Ehrenberg, Eyring, Giese, Harvey, Heinrich, Heller, Johnson, Kanda, Kishitani, Kluyver, Krukenberg, Mangold, McDermott, McElroy, Okada, Panceri, Phipson, Pierantoni, Pratje, Quatrefages, Spallanzani, Tilesius, Trojan, van der Burg, van der Kirk, van Schouwenburg, and Zirpolo. We regret that more of our contemporaries in this group were not present at the conference.

About 40 different orders of animals are self-luminous. These include such diverse organisms as bacteria, at least two groups of fungi, radiolaria, dinoflagellates and cystoflagellates; sponges, hydroids, medusae, siphonophores, pennatulids, ctenophores, and nemerteans; 7 families of marine worms, earthworms; ostracod, copepod, decapod and schizopod crustaceans; myriapods, possibly spiders; spring-tails, flies, and beetles; brittle-stars, bivalves, nudibranchs, and two orders of squid; balanoglossids, ascidians, and several orders of fish, both elasmobranchs and teleosts. The phyla Platyhelminthes, Nemathelminthes, Trochelminthes and all vertebrates above the fish (amphibians, reptiles, birds, and mammals) are non-

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luminous. All higher plants (Bryophytes, Pteridophytes, and Spermatophytes) also contain no luminous forms.

Among the luminous groups listed above, only terrestrial or marine animals produce light, while fresh-water organisms do not, even though closely related to luminous marine species. The nearest approach to a luminous fresh-water animal is an aquatic firefly larva breathing by

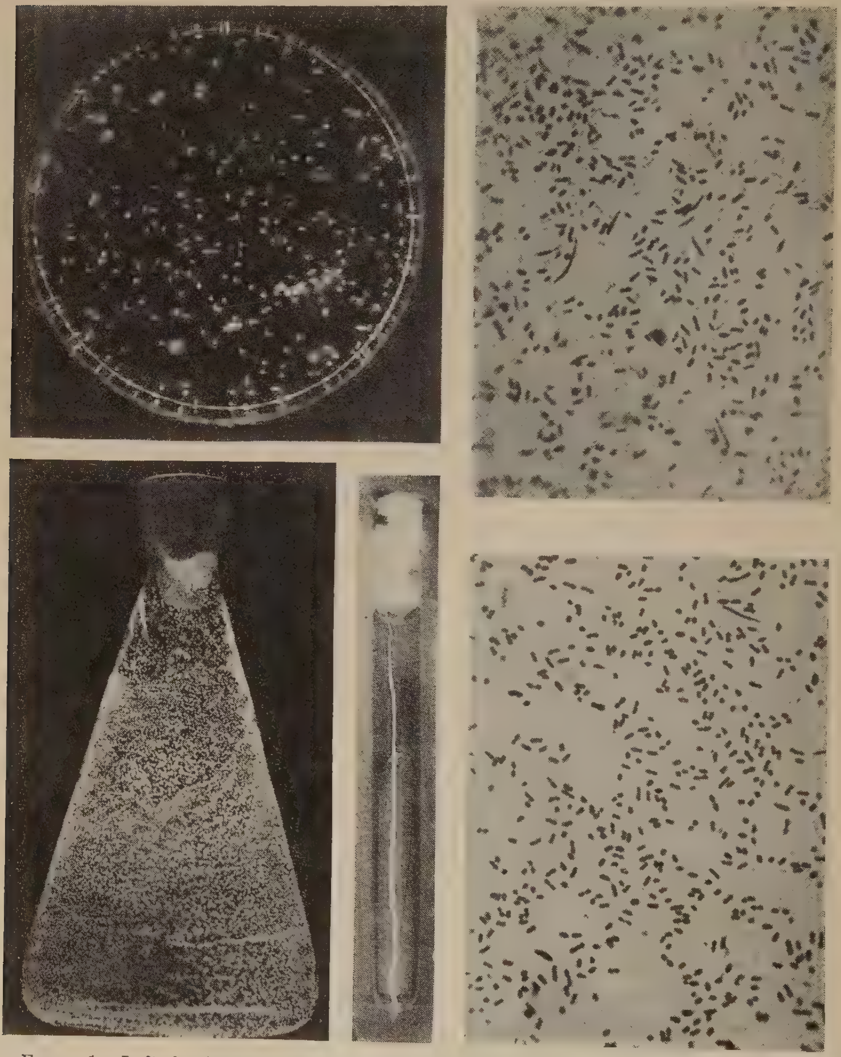

FIGURE 1. Left, luminous bacterial colonies growing on a Petri dish, flask, and test tube of culture medium (after Molish). Right, luminous bacteria highly magnified, *Pseudomonas toyamensis* (above) and *Coccobacillus ikiensis* (below), after Kishitani.

tracheal gills. The diverse and apparently chance distribution of luminescence in the living world suggests that the mechanism for producing light has developed in the course of evolution from some one of the chemical systems already generally present in cells and probably from one concerned with cell respiration.

Hence, much is to be gained from a study of luminescence in organisms such as luminous bacteria, where the cell respiration is intimately connected with the production of light. These bacteria occur in the sea and frequently form colonies on dead fish or squid and also on meat in refrigerators. They are of several species, are non-pathogenic to humans, and easy to culture on 2 per cent peptone, 1 per cent glycerine sea water agar at room temperature or below. A pinch of $CaCO_3$ can be added to maintain the proper pH. A good idea of their appearance can be obtained from FIGURE 1.*

In addition to such saprophytic bacteria, there are also parasitic forms. Various organisms, such as sand fleas, shrimps, midges, or caterpillars may become spontaneously infected with parasitic luminous bacteria and develop a luminous malady that is finally fatal. In the meantime, the host animals move about and would be mistaken for true luminous organisms if the origin of the light were not known.

Finally, certain squid and fish may be occasionally or regularly luminous because of the harboring of luminous bacteria in their glands. No harm results to the host from these bacteria. In the remarkable cases of the East Indian fish, *Photoblepharon* and *Anomalops* (shown in FIGURE 2), a special light organ has been developed under the eye in which luminous bacteria are always present. They live between special long cylindrical cells which are richly supplied with blood capillaries. Moreover, the continuous light emission of the bacteria can be shut off by a screening mechanism of the fish. In *Photoblepharon,* there is a fold of black membrane, like an eyelid, that can be drawn up over the organ, thus obscuring the light. In *Anomalops,* the light organ is attached at the anterodorsal corner by a hinge that allows the whole organ to be turned over and downward into a black-pigmented groove or pocket, so that none of the light-emitting surface is visible.

These two fish alone present enough problems for the evolutionists. Why should these two very closely related genera have developed two totally different methods of shutting off the light? When do the bacteria get into the organ? Nothing is known about the embryology of the fish or why so large a light surface should appear immediately under the eye. Such problems might warrant an expedition to the Banda Sea, the only

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* FIGURES 1-4 are reproduced from *Living Light* by E. Newton Harvey, courtesy of Princeton University Press.

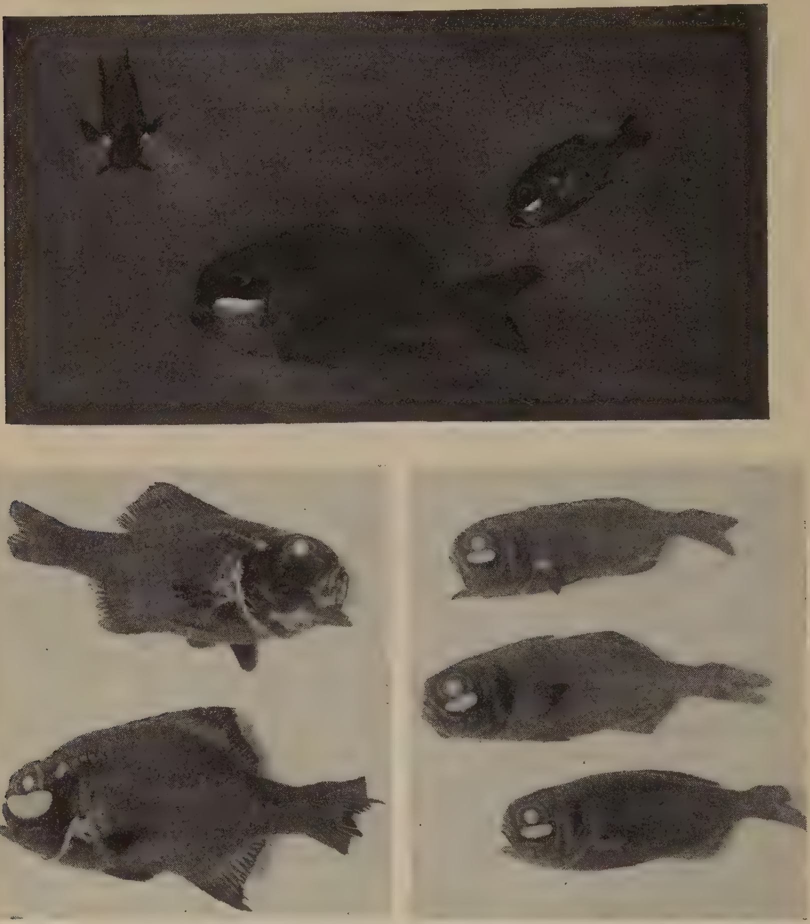
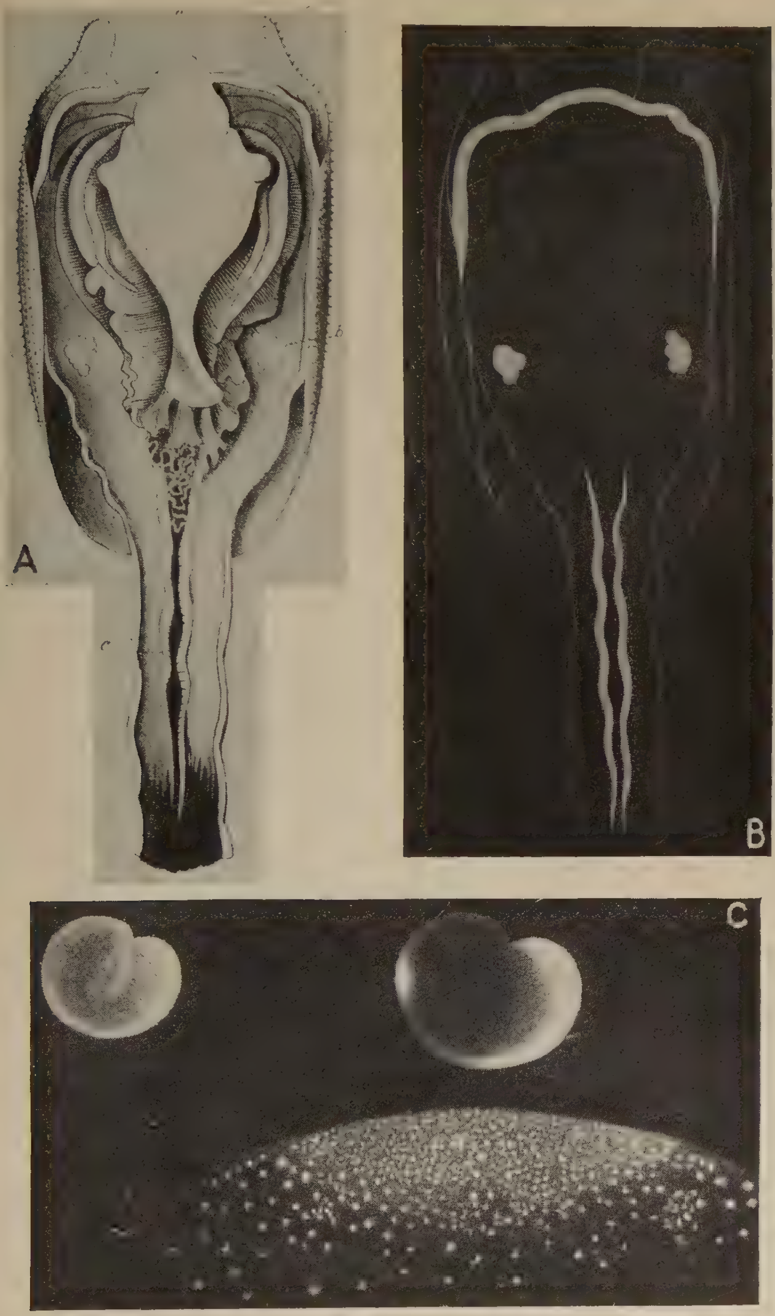


FIGURE 2. Above, the fish, *Photoblepharon palpebratus*, swimming in water of the Banda Islands at night. (After Dahlgren, from a painting by Bruce Horsfall.) Below, *Photoblepharon palpebratus* (left) and *Anomalops katoptron* (right), photographed from dead specimens, showing the large luminous organ, under each eye, whose light comes from symbiotic luminous bacteria.

(*See opposite page*)

FIGURE 3. A. *Pholas dactylus*, the luminous clam with which Raphael Dubois demonstrated the luciferin-luciferase reaction, in daylight. B. The same at night to show the luminous regions (after Panceri). C. The flagellate, *Noctiluca miliaris*, showing luminescence of two whole organisms and the appearance under the microscope, where the light can be seen to come from small discrete luminous granules (after Quatrefages).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C

place in the world where *Photoblepharon* and *Anomalops* occur in considerable numbers.

Bioluminescence is actually a chemiluminescence, *i.e.*, light production during a chemical reaction. It is quite fitting, therefore, that a conference on bioluminescence should begin with light production by pure organic substances in solution. Many such compounds are known and the light emitted by some is indeed brilliant. Examples of these chemiluminescences will be discussed by Dr. Anderson.

The chemiluminescences responsible for the light of organisms involve the oxidation of a compound, luciferin, in presence of an enzyme, luciferase. In some organisms (fireflies and bacteria) the oxidation is intracellular; in others (*Cypridina* and *Pholas*) extracellular, a large amount of luminous secretion being stored in a special gland. In certain organisms (bacteria, protozoa), a single cell may produce the light. In others (shrimp, squid, fish), accessory structures have been developed, so that the light organ can be truly described as a lantern, with lens, reflector, and sometimes both color and opaque screen. In the bacteria and fungi, the light emission is continuous day and night; whereas in all other forms, it appears only on stimulation.

Success in the study of any biological phenomenon is dependent on particularly favorable experimental material, *i.e.*, what we have come to speak of as classic forms. A good example of classic organisms for study are the luminous bacteria which have already been mentioned. Their contribution to a study of bioluminescence will be discussed by Dr. Johnson. Unfortunately, no one has as yet succeeded in extracting luciferin and luciferase from luminous bacteria. These substances, first demonstrated by Raphael Dubois in the elaterid beetle, *Pyrophorus*, in 1886, and later obtained from the mollusc, *Pholas dactylus*, and studied in detail by Dubois, are best extracted from luminous animals with extracellular luminescence, where a large quantity of luminous secretion is formed. *Pholas* is pictured in FIGURE 3A and B.

In 1916, during a trip to Japan for the collection of luminous squid, I noticed the abundant luminous secretion of the small ostracod crustacean, *Cypridina hilgendorfii*, and was at once able to establish the presence of luciferin and luciferase. These substances are secreted from separate, long, single, gland cells opening at pores near the mouth. Moreover,

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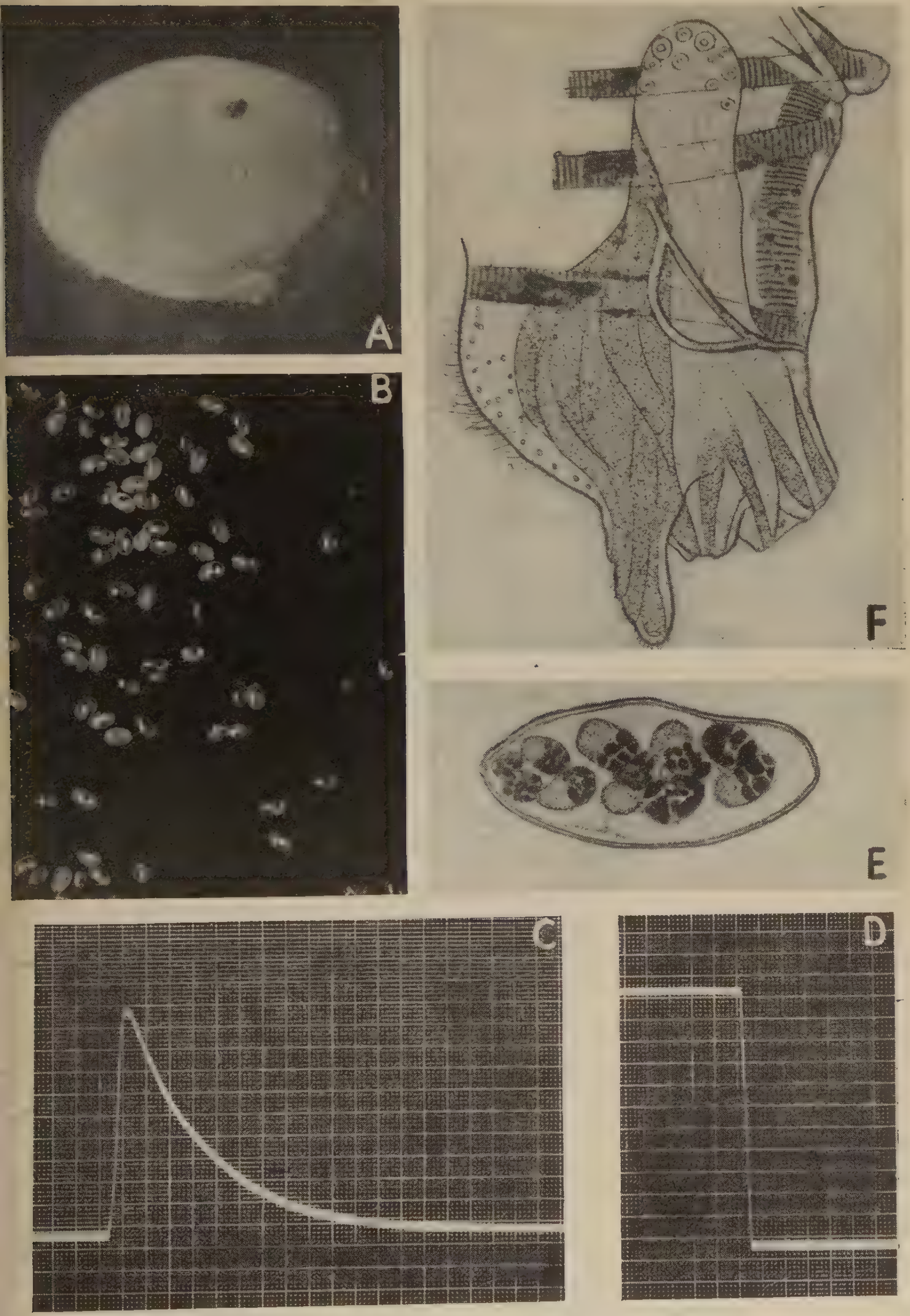
(*See opposite page*)

FIGURE 4. A. A single ostracod crustacean, *Cypridina hilgendorfii*, enlarged, showing the black eye-spot, tip of swimming legs (below) and protuberances of luminous gland (right). B. Dried *Cypridinae*, life-size, photographed on a cloth. C. A photo-cell and string galvanometer record of light, intensely (vertical), as a function of time (horizontal), when *Cypridina* luciferin and luciferase are mixed. D. A similar record of ink mixed with water to determine time of mixing. Light intensity in arbitrary units. Each large division represents 0.2 seconds. (After Harvey and Snell). E. A cross-section of the gland of *Cypridina*. F. A longitudinal section of the gland region, showing two types of single gland cells, opening by separate pores, and cross-striated muscle fibers, whose contraction squeezes the secretion into the sea water (after Yatsu).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the whole animal, less than an eighth of an inch long, can be obtained in fair quantities and, when dried rapidly, indefinitely retains the ability to luminesce whenever moistened. Such dried material has been invaluable in a study of the chemistry of luciferin, which, together with some observations on *Cypridina* luciferase, will be presented by Dr. Chase. *Cypridina,* shown in FIGURE 4A and B, has become the classic organism for chemical study of bioluminescence. The histology of the gland is pictured in FIGURE 4E and F, and a record of *Cypridina* luminescence in FIGURE 4C and D.

One of the almost universal characteristics of luminous forms (except for the bacteria and fungi) is the ability to flash on stimulation. The effect is particularly well seen in the "phosphorescence of the sea", where waves dashing on shore, the splash of oars, or the wake of a boat are outlined by the flash of thousands of small marine organisms, mostly flagellates, stimulated mechanically by the motion of the water. *Noctiluca,* the best known of the flagellates, is shown in FIGURE 3C.

Higher organisms, also, possess a mechanism for turning their light on and off, well seen in the firefly flash. In fact, fireflies are classic material, not only for study of the physiological problem of flashing, but also for investigation of the fine histological details of luminous organ structure, which is necessary for an understanding of the physiology of flashing. Dr. Buck will consider these aspects of the bioluminescence problem and will also, I hope, describe his ecological studies on the firefly and the use of the flash as a mating signal. We need a scientific explanation of the use of the light in many luminous animals as well as of the mechanism of cold light emission itself, that unique phenomenon which, in the form of the fluorescent lamp, is now used to light our homes.

CHEMILUMINESCENCE IN AQUEOUS SOLUTIONS

By Rubert S. Anderson

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In trying to analyze a biological process such as bioluminescence, a beginning is often made by relating the biological observations to the most similar parts of pure chemistry and physics. This has been done in connection with light given off by living organisms or by extracts from them, and at least the most common type of luminescence has been generally agreed upon. In the case of the best known system, *Cypridina* luciferin and luciferase, the light is clearly the result of a chemiluminescence. Even the reaction producing it, an oxidation by oxygen, is a common type of reaction producing chemiluminescence. The bioluminescence of many forms seems to be similar, in various ways, to that of *Cypridina,* and these forms are therefore believed to contain chemiluminescent systems even though they may never have been separated from the cell.

It would be helpful if there existed a well developed body of information about the chemiluminescence of known compounds in solution. Unfortunately this is not the case, and answers to most of the questions which might arise in the study of bioluminescence cannot be given. It is the purpose of the present review to collect a number of the many scattered observations made, particularly in recent years, on chemiluminescence in water solutions, with little reference to the biological systems.

From early times, the characteristic of bioluminescence and chemiluminescence which has seemed most striking has been light-emission at ordinary temperatures, in contrast to its usual association with high temperatures. This property is also shown by fluorescence. The actual emission process is probably analogous. It results, most immediately, from loss of a quantum of energy during the change of an excited electronic state to the ground state or some other state having less energy than the initial excited one. The major difference between chemiluminescence and fluorescence is, then, the means by which the excited electronic state is first produced. In a chemiluminescent reaction, a part of the energy resulting from the reaction is channeled in a very particular way instead of being dissipated as heat. In fluorescence also, the energy is introduced into the molecule in a specific way, *i.e.,* by the absorption of radiation. Neither the emission of the radiation, nor the production of the excited state, need depend on a high temperature.

Even though excited states have been produced, in the one case by a chemical reaction and in the other by absorption of radiation, the excess energy need not be lost as radiation. While some of the molecules having the excess energy may emit light, others may lose it as heat or in chemical reaction. The ratio of the number of light emitters to those experiencing energy loss in other ways can be modified by various means, particularly by the addition of so-called quenching agents, which decrease the amount of light. Quantitatively, light emission at ordinary temperatures is a relatively uncommon process. For instance, absorption of light, capable of raising electrons to excited states, occurs at least in all colored solutions. Yet many colored solutions are not fluorescent at all and, of those that are, the efficiency rarely approaches one.

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The original production of the excited state from a chemical reaction is supposed to result from a crossing or near approach of potential energy surfaces. Eyring *et al.*[1] also consider that it may occur as a result of direct excitation by black body radiation, at least in the case of some extremely faint instances of chemiluminescence studied by Audubert.[2] The crossing or near approach of potential energy surfaces may perhaps be visualized in some way such as the following. If, for a compound, some configuration of the nuclei exists, a distance in the diatomic case in which the system has approximately the same potential energy with its electrons in the ground state and in an excited state, a shift may occur from the ground state to the excited state. The system may be brought to this critical configuration by a chemical reaction. When the configuration changes, the electron excitation energy is trapped and may then be lost as a quantum of light.

Considering, now, specific systems, it was stated above that no systems in solution are well understood. In fact, until fifteen or twenty years ago no reactions of specific compounds in aqueous or other solvents were known which gave off as much light for comparable amounts of material as the extracts from organisms. Lophine and the Grignard compounds probably approached them most closely. That is no longer true, since the description of the bright chemiluminescence of aminophthalhydrazide by Albrecht in 1928[3] and of dimethylbiacridinium nitrate by Gleu and Petsch in 1935.[4]

To find a chemiluminescent reaction which can be described with some completeness, it is necessary to turn to a much simpler system, a gas reaction. Gas reactions seem remote from bioluminescence or even chemiluminescence in solution, but they may act as a guide in a number of ways, and some of the same general experiments apply. In 1928 Polanyi and his co-workers reported extensive experimental work on the reaction of the alkali metals with several halogens and halides. For our purpose, it is necessary to consider only one of these reactions, that between sodium vapor and chlorine.[5] When these two elements react under the proper conditions, light is emitted. The sodium vapor is introduced at one end of a tube at a pressure of only 0.001 or 0.01 mm. Hg. The chlorine is introduced into the tube as a jet. The simplest condition is to have the sodium in excess. The reaction occurs where the two gases meet. From the character of the emitted light, the effect of pressure of reactants, the effect of foreign gases, and from the position of the luminescence or flame with respect to the deposit of reaction product (NaCl), the several steps of the reaction have been obtained. The following major events are believed to occur:

$$\begin{array}{lrcl}
(1) & \mathrm{Na} + \mathrm{Cl_2} & \rightharpoonup & \mathrm{NaCl} + \mathrm{Cl}. \\
(2) & \mathrm{Na_2} + \mathrm{Cl} & \rightharpoonup & \mathrm{NaCl^*} + \mathrm{Na}. \\
(3) & \mathrm{NaCl^*} + \mathrm{Na} & \rightharpoonup & \mathrm{NaCl} + \mathrm{Na^*}. \\
 & \mathrm{Na^*} & \rightharpoonup & \mathrm{Na} + \text{light}. \\
(4) & \mathrm{Na} + \mathrm{Cl} & \rightharpoonup & \mathrm{NaCl}. \\
(5) & \mathrm{NaCl^*} & \rightharpoonup & \mathrm{NaCl} + \text{heat}.
\end{array}$$

Most of the light which is emitted has been identified as the D-line of sodium. This immediately shows the emitting material to be primarily sodium atoms. The frequency of the D-line is equivalent to 48.5 Cals. Reaction 1 furnishes only 35 Cals., which is not enough to produce the light emitted. Reaction 2, giving 75 Cals., provides more than the necessary energy for the emitted light and is believed to be its source. That the sodium atom produced in reaction 2 is not excited as it is produced, follows from a number of additional facts. The chemiluminescence of the reaction is quenched by foreign gases more strongly than the resonance emission of the D-line of sodium. This means that the excess energy is retained long enough for collisions to occur and dissipate the energy in some other way. The emission of the D-line by excited sodium occurs after about 10^{-8} seconds. It is, therefore, suggested by Polanyi that the excess energy is retained by some other material for a longer period than 10^{-8} seconds. This material is believed to be the sodium chloride. The excess energy of the sodium chloride molecules is then supposedly transferred to sodium atoms during collisions as indicated in reaction 3. The excited sodium atom thereupon loses the energy as light. Reactions 4 and 5 are competing non-luminescent reactions occurring at appreciable rates only at the wall. The very large effect of changes in the pressure of sodium vapor results from differential effects on the several reactions; that is, increased pressure of sodium favors reactions 2 and 3 as compared to 4 and 5, and an increased yield of light results. An increase of sodium from 0.001 to 0.01 mm. Hg. pressure increases the quantum yield, per two atoms of sodium reacting, from about 1 per cent to about 35 per cent. The calculated maximum quantum efficiency with still greater pressures of sodium was set at about 0.85, although this was not realized experimentally.

A fairly complete description, therefore, can be given of the chemical reactions leading up to the emission of the light and the side reactions which compete with those responsible for chemiluminescence. Even in this apparently very simple case, quite a number of complicating factors are present. In view of these complications in a simple gas reaction, it is not surprising that the description of events in the reaction of a large organic molecule in solution is so inadequate. However, although some of the features of these gas reactions are special to them, the general experimental approach through spectral emission curves, concentrations of reactants in relation to light emitted, quenching, etc., are applicable to studies in solution.

The number of known chemiluminescent reactions in solution is large, although this number is a very small fraction of the total known reactions. Harvey, in his book[6] indicates many of these reactions. Oxidation is a usual type of reaction, and pyrogallol, lophine, and the Grignard compounds are some of the better known materials undergoing reaction. A number of common systems previously considered to be non-radiating have been studied by Audubert.[2] By the use of specially designed counters, he reports finding very low intensities of ultraviolet light associated with many reactions.

This discussion will omit those reactions which show a very low efficiency and consider the two which seem most comparable, in amount of light emitted, to the gas reaction and to the bioluminescences. This distinction is, of course, arbitrary to some extent, since an intense chemiluminescence or fluorescence may be extinguished by a change of conditions. Recognizing this, it still seems that for some experimental purposes more emphasis should be placed on the quantitative differences between various reactions under the most favorable conditions known. In most of the known systems, the actual fraction of the total number of reacting molecules which give off light is extremely small. The fact that they have been observed at all is due in many examples to the extreme sensitivity of the eye as a detecting instrument. It may not be a case even of one in thousands or tens of thousands, but one in millions of molecules. In the reactions studied by Audubert, only one in 10^{14} or 10^{15} of the molecules which reacted emitted radiation. The aminophthalhydrazide[3] and the dimethylbiacridinium compounds[4] mentioned earlier are, in aqueous solutions, the most efficient light-producing systems which have so far been discovered. Each will be considered in some detail. Although a considerable study has been made of these reactions, definitive results upon which all authors are in agreement have not appeared.

The Phthalcyclohydrazides

Albrecht,[3] in 1928, studied extensively the chemiluminescence of several phthalcyclohydrazides of which the brightest and most studied was 3-aminophthalcyclohydrazide.* This hydrazide emits a blue light when it is oxidized in alkaline solution with a number of oxidizing agents such as hydrogen peroxide, potassium ferricyanide, or sodium hypochlorite. Convenient methods of preparing the hydrazide for demonstrating the reaction[7] and additional studies of related compounds[8] appeared a few years after Albrecht's work.

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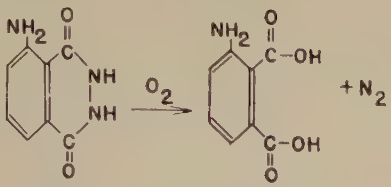
* Also called luminol and, by Chemical Abstracts, 5-amino-2,3-dihydro-1,4-phthalazinedione.

Albrecht formulated the overall reaction as:



The time course of the reaction is markedly dependent upon the conditions and especially upon the oxidizing agent which is used. Albrecht[3] found that, with most oxidizing agents, the light is dim and of short duration. With hydrogen peroxide, the reaction lasts for a much longer time although the light intensity is low. The intensity of the light can be much increased, although the duration is decreased, by having present in addition to the hydrogen peroxide, ferricyanide, hypochlorite, or one of a number of compounds which appear to act catalytically. In general, hydrogen peroxide in the medium is necessary for the large yields of light. Other authors[6, 10–20] have subsequently studied a variety of catalysts. Manganese dioxide, colloidal platinum, hemin or hemoglobin, many metal complexes, and ozone are a few of the materials which produce increased light intensity. Weber and his collaborators more recently have studied the relation of other trivalent iron complexes,[21] copper complexes,[22] $RuCl_3$ and $VOSO_4$[23] to the chemiluminescent reaction. Whether the compound is to be looked upon as a reactant or a catalyst depends, according to these authors, upon its rate of reoxidation by atmospheric oxygen.[21]

The form of the relation between intensity of light and time depends on the catalyst used. For instance, Weber *et al.*[21] consider that the light intensity decreases exponentially with time with a number of iron catalysts. Stross and Branch,[24] using hydrogen peroxide and ferricyanide, find the curve of log intensity against time to be convex upward. They also observe that the initial point at 0.2 seconds lies below the smooth curve and suggest that the luminescent reaction may not be the initial one. The intensity-time relation of the uncatalyzed, and hence slow, reaction of hydrogen peroxide with the hydrazide depends on the concentration of hydrogen peroxide and alkali.[25, 26] Many such curves show a point of inflection because the intensity of luminescence does not decrease as rapidly during the initial portion of the reaction as it does later.

It has long been known[3, 10] that the hydrazide would show visible luminescence at extremely great dilutions, one part in 10^8 parts of water. The quantitative efficiency of the light emission in terms of amount of light per mole of hydrazide present has also been studied. Harris and Parker,[27] by means of a flowing system, studied the light emitted by the hydrazide in a medium of 0.35 M sodium hydroxide, hydrogen peroxide, and sodium hypochlorite. The reaction was so fast that it was confined to a spot of light even in the flowing system. They found the efficiency to be markedly sensitive to the concentration of hydrogen peroxide, with the best condition approximately 4 moles of hydrogen peroxide per mole of hydrazide. Under their optimum conditions, from 0.003 to 0.005 as many quanta of light were produced as there were hydrazide molecules present, assuming that all of the light was emitted at 4250 Å. Use of potassium ferricyanide instead of the hypochlorite gave a more diffuse spot of light, presumably because the reaction rate was slower.

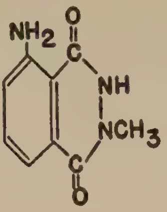
Stross and Branch[24] later published a study of the hydrazide also in a flowing system. They used varying concentrations of sodium hydroxide, hydrogen peroxide, and potassium ferricyanide. Instead of studying the light emission from a spot, they measured the light intensity at varying distances from the point of mixing. The maximum number of quanta observed per molecule of hydrazide, assuming that the light was emitted at 4570 Å, was 0.36. This is 50—100 times as great as the value given by Harris and Parker. Stross and Branch do not refer to the earlier work, so that no explanation of the discrepancy is available, apart from the different oxidizing agents.

The emitted light covers a broad region of wavelengths with a suggestion of a double maximum.[3, 28] The precise location of the curve is variously reported and may depend on the oxidizing agent or catalyst used with hydrogen peroxide. For instance, Eymers and Van Schouwenberg[28] report the maximum at 4400—4600 Å with hemin, while with ferricyanide the emission extends farther into the blue.

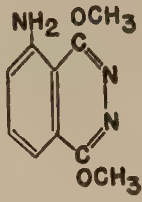
The emitted light, unlike the gas reaction of sodium and chlorine, gives no immediate clue as to the identity of the emitting molecule. An attempt has been made, however, to obtain some information by comparing the chemiluminescence and the fluorescence of the hydrazide and other compounds. When the fluorescence emission curve of a compound is similar to the chemiluminescence emission curve, it has been considered as evidence supporting the idea that this same compound also emits the light in the chemiluminescent reaction. Although it is not certain that the same molecule producing the two types of luminescence would necessarily emit identical spectral distribution curves, it has been considered a plausible assumption with which to begin. Such a comparison was first made by Albrecht.[3] An immediate complicating factor appears for the hydrazide. The chemiluminescence occurs best in alkaline solution, while the fluorescence appears in neutral or acid solution. Albrecht considered the two spectral distribution curves to be similar, even though the maxima were about 200 Å apart. This difference he ascribed to the different pH's of the solutions. His view was supported, in part, by Sveshnikov,[25] who brought the maxima closer together by bringing the pH's of the chemiluminescent and fluorescent solutions together more closely. The comparison of fluorescence and chemiluminescence of the hydrazide has been studied also by Eymers and Van Schouwenberg,[28] who believe that the two curves are essentially different. A comparison has also been made by Albrecht of the chemiluminescence and fluorescence of other phthalcyclohydrazides. He stated that the color of the emitted light shifted in a parallel manner in a series of derivatives. Whatever view may be the correct one, most discussions of the actual chemiluminescent reactions assume that the emitting molecule is the initial hydrazide itself or some closely related compound.

It is a matter of practical and perhaps of theoretical importance to know if the emission curves, as observed experimentally, represent the emitting molecule. Distortion could occur by the presence of compounds which either absorb some of the emitted light or fluoresce under its influence. According to the published curves,[25, 27, 29, 30] absorption by the hydrazide itself should not greatly influence the emission in the visible region, although the same may not be true for other reactants and catalysts which have been used. That fluorescence of various dyes occurs when they are dissolved in a solution of the hydrazide emitting chemiluminescence, has been shown by a number of authors.[31–33] Also, mixtures of hydrazide with dimethylbiacridinium salt, in solutions where only the hydrazide would show chemiluminescence, give the green light of the acridinium chemiluminescence or fluorescence.[34] Tamamusi[35] has favored the idea that such light emissions may not be true fluorescences but actual transfers of energy by collision of hydrazide with dye followed by light emission from the dye. A transfer of energy during collision of sodium chloride and atomic sodium is included in the gas reaction described above. However, no similar process has been shown for chemiluminescence in solution and, according to Weber and Ochsenfeld,[36] the experimental results on hydrazide can be quantitatively explained without assuming such an energy transfer.

The relation of structure to the luminescence has been clarified by work from a number of laboratories.[3, 9, 10, 37–45] Several conclusions were summarized by Drew[41] in the 1939 Faraday Symposium on luminescence, although agreement is not complete.[42, 43] According to Drew, the 6-membered ring hydrazide is an essential requirement for chemiluminescence. Neither the open chain nor the 5-membered ring hydrazides show any luminescence. Apparent exceptions to this rule are explained as due either to a preliminary change into the 6-membered ring compound or to the presence of traces of it as an impurity. It has also been found[9, 39, 40] that methyl group substitutions such as



or



give non-luminescent compounds. Therefore, it is concluded that both hydrogens must be present on the nitrogen atoms or as the di-enol. The sensitivity of the eye to light requires exceptionally rigorous purification of the compounds studied to avoid misinterpreting the data. Drew and Garwood[40] used as many as eight recrystallizations to free some materials of traces of chemiluminescent impurities. More recently, Huntress and Gladding[45] have shown that changing the order of the carbon atoms and nitrogen atoms in the ring in the several possible ureas and quinoxalines, results in compounds which do not luminesce although they appear to oxidize.

Given the 6-membered ring hydrazide, Albrecht[3] showed in his thesis that substituents in the other ring greatly influenced the amount of light emitted. An amino group in the 3 or 4 position increased the amount of light as compared with the unsubstituted phthalhydrazide. Position 3 was more effective than 4. The nitro group decreased the amount of light. Drew[41] concluded that, in general, substitutions in the *ortho* positions with respect to the cyclohydrazide ring had greater quantitative effects than the corresponding substitutions in the *meta* positions. The nature of the substituent in the benzene ring has a great effect on the intensity of the light emitted and also influences its color.[3, 38] Some relative intensity measurements have been made[38, 40, 42] which permit a listing of substituent groups in the approximate order of light emitted. Groups such as NH_2, NHMe, OH, $NH \bullet NH_2$, in that order, and to a lesser extent chlorine, bromine, and iodine, increase the amount of light emitted as compared to the unsubstituted compound. On the other hand, groups such as NO_2 increase the light very little or decrease it. In general, Drew and Pearman[38] consider the first class to correspond to the *ortho* and *para* directing groups of organic chemistry, and the second to *meta* directing groups.

It may be that this apparent relation between the amount of chemiluminescence and the directive influence of substituent groups comes about through the usual effect of the groups on the chemical reaction. In this connection, however, some results obtained by West[46] on the fluorescence of naphthalene and its derivatives are of interest. The following relative efficiencies of fluorescence are reported:

NH_2	: 1
β-OH	: 0.75
β-CN	: 0.5
α-COOH	: 0.3
Naphthalene	: 0.15
α-chlor	: 0.05
NO_2	: none.

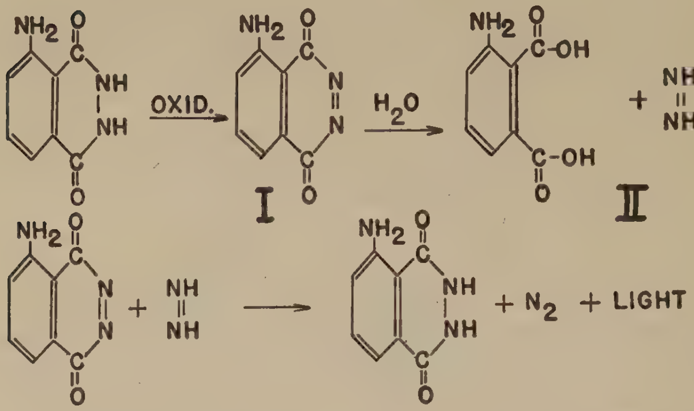
Although these results presumably do not involve a chemical reaction, the various groups again have a striking and specific effect on the quantitative result. Also, the order of the groups is approximately the same as for the chemiluminescence, with the exception of cyanide and carboxyl which are not given by Drew and Pearman.[38] These two are *meta* orienting groups.

Compounds present in solution also have a marked influence on the amount of chemiluminescence, just as in fluorescence. For instance, it has been found that traces of hydroquinone[24, 42] cause a great diminution in the light intensity. A series of papers by Weber and his collaborators have reported studies on the influence of many compounds and the halide ions on the fluorescence[47] and chemiluminescence[21-23] of the hydrazide. The influence on chemiluminescence may operate both through the chemical reaction and through quenching, which is analogous to fluorescence quenching. That the effect of chloride and bromide ions on a chemiluminescent reaction need not be to decrease but may increase the amount of light emission, has been shown with the *Cypridina* luciferin and luciferase system.[48]

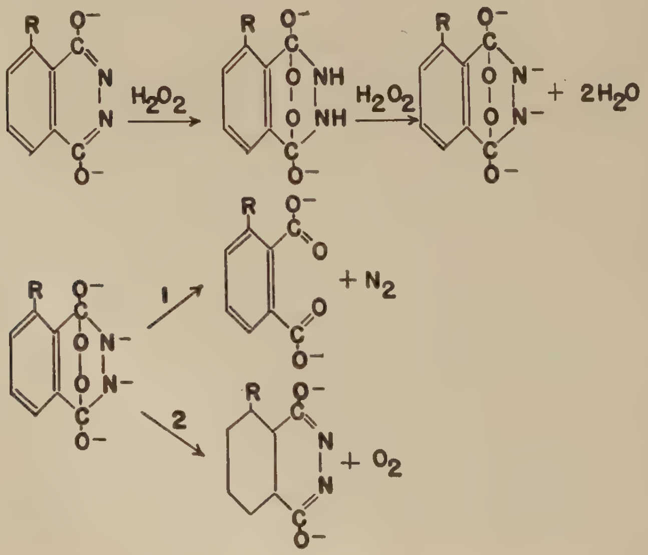
The actual reactions which produce the chemiluminescence are not agreed upon, although several authors[3, 21, 24, 41, 49] have suggested systems which account for a certain amount of the experimental data. Albrecht's[3] original formulation (see following page) has not been accepted.[41] The azodiacyl compound (I) is unknown, and the diimine (II) is hypothetical. However, in 1942, the finding of a compound, colored and unstable, believed to be (I) was reported by Kautsky and Kaiser.[50] A solution of this material, free of oxidizing agent or oxygen, is said to emit light when made alkaline. No further work on its isolation and identification has appeared.

Meanwhile, Drew and Garwood[40] reported the isolation of a sodium salt of the peroxide of the 3-aminophthalcyclohydrazide. This compound, dissolved in water, gives off luminescence when hemoglobin is added to the solution. The intensity increases when alkali is added.

The same authors also report that the extent of the destruction of the hydrazide during chemiluminescence depends on the conditions, includ-



ing particularly the oxidizing agent used. Destruction, with evolution of all of the hydrazide and much of the amino nitrogen, occurs with hypochlorite and permanganate. However, when to a solution of hydrazide and hydrogen peroxide, sodium hypochlorite was added slowly, as little as one-seventh of the hydrazide nitrogen was liberated. The authors suggest, therefore, that most of the hydrazide can be recovered unchanged and that the destruction of this ring may be a side reaction and not an integral part of the chemiluminescent reaction. Their provisional formulation is as follows, on the assumption that the hydrazide in alkaline solution is in the enol form and ionized although no titration curve is presented:



Reaction 1 leads to the release of nitrogen and need not be luminescent. Reaction 2 may be the major source of luminescence. This, unlike most previous ideas, suggests that the hydrazide is not the substrate but a catalyst for the decomposition of hydrogen peroxide, and it has been so considered in some kinetic studies.[25] A study of the reactions of the isolated hydrazide peroxide, including quantitative determinations of nitrogen and oxygen, should be revealing.

Stross and Branch,[24] also in 1938, on the basis of quantitative studies on the reactants, ferricyanide and hydrogen peroxide, and accounting for the oxygen, concluded that the chemiluminescence was associated with a two-step oxidation, probably to the azo compound postulated by Albrecht.[3] In contradiction to Albrecht and in agreement with Drew,[41] they consider that the luminescence comes before the breaking of the hydrazide ring. They consider that in their reaction system the ferricyanide produces a one-unit oxidation to a free radical which then reacts with hydrogen peroxide.

Additional proposals and experiments have been contributed by others.[21, 25, 49] Weiss[49] combines a number of chemiluminescences into a single scheme. Lophine, after hydrolysis, luciferin, and the hydrazide have fairly labile hydrogen atoms which, he states, can be removed by oxidation. In general, Weiss says that the reaction can be written, $AH_2 \rightarrow AH \rightarrow A$, where A represents all of the compound except the two labile hydrogen atoms. This formulation also has the free radical, AH, as an intermediate step. The actual luminescence arises, according to Weiss's theory, by combination of radicals or ions:

$$2\ AH \rightarrow AH_2 + A^*$$
$$\text{or, } AH \rightleftarrows A^- + H^+ \text{ and } AH + A^- \rightleftarrows AH^- + A^*.$$

Weber and his collaborators[21] include both the peroxide of Drew[40] and Albrecht's[3] system in a combined series of reactions.

It is evident that the problem has been left in a confused state, perhaps because of the war. Most authors, except Drew, include the azo compound to which Kautsky has suggested that he has a direct approach, but for which he has published only fragmentary evidence. Drew's isolation of the peroxide, on the other hand, seems definitive, but limited qualitative and quantitative data have been published upon its relation to the luminescent reaction, action of catalysts, etc. All formulations seem highly provisional until these basic matters and the fundamental position of the hydrazide in the luminescent reaction are settled.

The Biacridinium Salts

Gleu and Petsch,[4] in 1935, showed that N,N′-dimethylbiacridinium nitrate* gives a chemiluminescence when treated with hydrogen peroxide in alkaline solution. The chemiluminescence is green, as is the fluorescence. The compound is yellow. The intensity of the light emitted varies widely, depending upon the conditions. In solutions made alkaline with sodium hydroxide, the luminescence is relatively dim when hydrogen peroxide is added, but it lasts for a long time. The intensity can be increased enormously by the addition of osmium tetroxide as a catalyst and the time of luminescence decreased to one second. Under such conditions, light could be observed at a concentration of luminescent substance of 10^{-10} M. When the reaction is carried out in a medium of concentrated ammonium hydroxide, the light with hydrogen peroxide alone is much brighter than in a sodium hydroxide solution. It may then last for only a minute. Osmium tetroxide has less effect on the reaction in concentrated ammonia. The quantitative effects of temperature,[51] the kinetics of the reaction,[25, 26] and the quenching or enhancing effects of foreign substances[36, 52] have been studied.

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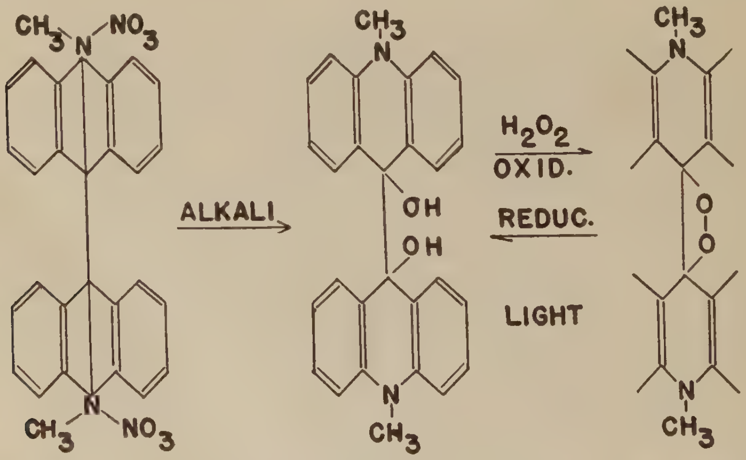
* Also called lucigenin.

Unexpectedly, when Gleu and Petsch tried other oxidizing agents, they observed no luminescence. However, they found that a number of common reducing agents, such as hydrosulfite or stannite, produced a chemiluminescence if oxygen was also present. None was observed if the solution was first freed of oxygen by passing nitrogen through it. In view of the need for oxygen in order to obtain luminescence, the authors concluded that the reaction was both an oxidation and a reduction. Since hydrogen peroxide is both an oxidizing and a reducing agent, this was not inconsistent with the other findings.

A tentative formulation designed to explain these several facts was given as follows:



According to this idea, the biacridinium salt would function essentially as a catalyst. With hydrogen peroxide, the net reaction would be:

$$2\ H_2O_2 \rightarrow 2H_2O + O_2 + 46\ \text{Cals.},$$

and with the other reductants, the oxidation of the reducing agent by atmospheric oxygen. One difficulty immediately arises, since the 46 Cals. are about 10 Cals. too small for some of the wavelengths represented in the light emission. However, Gleu considers that this does not definitely prove the reaction to be inadequate.

Comparisons of the spectral distribution curves of fluorescence and chemiluminescence have also been made for the biacridinium salts. The compound fluoresces in water and in acid solution. According to Weber, it will also fluoresce in mildly alkaline sodium carbonate solutions but, like the hydrazide, the chemiluminescence and fluorescence appear to have widely different pH optima. The original dimethylbiacridinium compound was studied by Eymers and Van Schouwenberg,[28] who concluded that the fluorescent and chemiluminescent spectra were identical, even though the media in which they were studied were not identical. These same authors have extended their work by comparing fluorescent and chemiluminescent spectral distribution curves, including bioluminescent sources, after analysis. The analysis was based on the assumption that the observed graph, when intensity was plotted against frequency, was made up of two or more essentially symmetrical curves. The frequencies of these postulated fundamental curves tended to be the same for different reactions. How much the results depend on the above described secondary fluorescence is unknown.

More recently, however, Gleu and collaborators[53-55] have prepared a number of biacridinium derivatives and compared the color of the fluorescence and chemiluminescence.[55] The diethyl derivative shows identical green fluorescence and chemiluminescence. The same is true for the diphenyl at some concentrations. However, below 10^{-6} M, the chemiluminescence becomes blue. Fluorescence and chemiluminescence also differ in other compounds. The authors conclude, therefore, that the supposed agreement, which they and others had previously reported and used as a partial basis for the above formulation, is merely a coincidence which occurs with some compounds.

Kautsky and Kaiser,[50] in 1943, reported that all previous interpretations of the spectral distribution curve of the chemiluminescence are faulty. As ordinarily obtained, they consider the light emission to be a mixture of a true chemiluminescence and the green fluorescence of the biacridinium nitrate. They state that when the reaction is carried out in a dilute solution at 40—50° C., the fluorescence is largely absent and the true chemiluminescence, blue in color, is obtained. This seems to fit the observations of Gleu and Schaarschmidt[55] mentioned above. Further, Kautsky and Kaiser find that the spectral distribution curve of this "true chemiluminescence" is closely similar to the fluorescence of N-methylacridone under exactly the same conditions. It is the acridone, therefore, which these authors consider as being the primary emitter of the luminescence, and they regard its formation from the carbinol with liberation of 65 Cals. as the chemiluminescent reaction. If this is true, there should be a striking effect of pH on the color of the emission curve as usually obtained, since the fluorescence of the biacridinium salt is reported as decreasing in alkaline solution. Also, the question arises as to how the reducing agents produce their effect, since the chemiluminescent reaction, as formulated with hydrogen peroxide by these authors, is an oxidation.

Unfortunately, no additional work has been found, so that here, also, the fundamental role in the luminescent reaction, substrate or catalyst, of the major compound is in doubt. The formation of acridone as a possible side reaction was mentioned by Gleu and Petsch.[4]

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THE CHEMISTRY OF *CYPRIDINA* LUCIFERIN

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Luminescence is encountered throughout the animal kingdom and in certain lower forms of plant life. However, the luminescent reaction has been demonstrated *in vitro* in only five orders of animals. These include certain beetles (fireflies), one mollusc (*Pholas*), certain ostracods (*e.g., Cypridina*), a few worms (*e.g., Odontosyllis*), and at least one decapod, a deep-sea form. The most favorable organism as a source of material for the study of the luminescent reaction *in vitro* is the ostracod crustacean, *Cypridina hilgendorfii,* whose eminent suitability for the extraction of the compounds concerned was pointed out by Harvey (1917). This animal possesses a gland in the head region, where luciferase, the enzyme which catalyzes the reaction, and luciferin, the substrate, are produced. When alive, the animal can eject these two compounds, in the form of two kinds of granules, into the sea water through separate pores, and a blue luminescence then occurs in the water. If the organisms are dried immediately after being caught, and are kept dry, the luciferin and luciferase in the gland remain stable for years and can be extracted when desired. The paper by Harvey (1948) contains photographs of *Cypridina* and drawings of the gland, on page 335.

The simplest method of extracting luciferin from *Cypridina* is by grinding up the dry organisms and adding hot water. This inactivates the luciferase, so that no luminescence occurs in the extract but the luciferin is obtained unaltered. The suspension is immediately cooled to retard destruction of the luciferin by oxidation. After filtering, the clear solution contains luciferin and all the other water-soluble components of the organism. Such crude luciferin extracts, though easy to prepare, are not very suitable for chemical work, because of the great instability of the luciferin in the presence of dissolved oxygen and of oxidizing systems that are extracted along with the luciferin. Also, the impurity of such preparations is undesirable from a chemical point of view and, indeed, luciferin in crude extracts often behaves quite differently than does more highly purified luciferin.

Several methods of extracting and purifying *Cypridina* luciferin have been developed. Kanda (1924, 1929) extracted the dry, powdered organisms with methyl alcohol in absence of oxygen and, after various precipitations and re-solutions, obtained luciferin which was more stable against oxidation and certainly purer than that in a crude aqueous extract. The chemical work on luciferin prior to about 1940 is discussed in publications of Harvey (1940, 1941).

The greatest advance toward the purification of luciferin is due to the work of Anderson. To determine quantitatively the concentration of luciferin present in his various purification steps, he developed a photoelectric method for measuring the total light emitted by a luciferin solution. This apparatus (Anderson, 1933) does not measure light intensity directly, as do most photoelectric and all visual methods, but instead it yields the integral curve for the luminescent reaction. The output of the photoelectric cell is stored in a condenser and the charge accumulated on the condenser, after any time from the start of the reaction, can be balanced with a potentiometer, using a Lindemann electrometer. The method is therefore a null-point one, and light-emission can be measured in terms of millivolts, to about one millivolt, the limit set by the Lindemann electrometer. The capacity of the condenser determines the sensitivity of the method. With this apparatus, very weak luminescences can be measured quantitatively. A good example of the sort of data obtainable is given in FIGURE 2. The value of the ordinate at any time is a relative measure of the amount of luciferin that has reacted with luciferase to give luminescence, from the start of the reaction to that time. The slope of the curve at any time is proportional to the intensity of the luminescence at that particular time. The data secured by the method are, therefore, analogous to those obtained in any reaction where an end product is determined quantitatively at various times during the course of the reaction.

Anderson (1935) worked out a purification procedure which yields from *Cypridina* a luciferin of much greater purity than had been obtained previously. Briefly, the method is as follows. Dry, powdered organisms are extracted for twenty-four hours with absolute methyl alcohol that is kept free of dissolved oxygen by saturation with purified hydrogen in a special extraction vessel. The vessel is then opened and a small amount of *n*-butyl alcohol is added and the methyl alcohol is removed by evaporation at low pressure. This solution, after having been chilled, is treated with benzoyl chloride. The resulting derivative of luciferin not only does not give light on addition of luciferase, but is much more stable in the presence of air than is the luciferin in its original state. Water is now added to hydrolyze the excess benzoyl chloride, and the butyl alcohol fraction is dissolved in 10 volumes of water. The resulting solution is extracted with ether, into which most of the butyl alcohol and luciferin derivative pass, leaving highly colored impurities in the aqueous phase. The ether is next removed *in vacuo,* leaving the inactive luciferin, already considerably free of colored impurities, in the residual butyl alcohol. This solution is mixed with a large volume of 0.5 N HCl, saturated with hydrogen, and heated in a hydrogen atmosphere for an hour at 95°-100° C and then cooled in an ice water bath. The mixture, now containing active luciferin, is then washed again with ether. At this stage, the "reactivated" luciferin remains almost entirely in the aqueous phase, and considerable colored material passes into the ether phase. The luciferin is finally extracted from the aqueous solution with *n*-butyl alcohol, deaerated with hydrogen and, if desired, is put through the same cycle of purification a second time. Some yellow color remains even after three cycles of purification and, as will be apparent presently, this is undoubtedly a property of luciferin.

This method results in a purification of the luciferin of about 2,000 times, in terms of amount of light per unit of dry weight of solid material in the final solution as compared with the starting material. The stability of the luciferin against oxidation is also greatly increased, perhaps because oxidizing systems that were present in the original material have been removed during the purification procedure.

In most of the recent work which has been done on the chemistry of luciferin, the luciferin has first been subjected to Anderson's (1935) purification procedure. It has frequently been found that quite different experimental results are obtained when using this purified material than with crude aqueous extracts of *Cypridina.* For example, luciferin in crude extracts is not affected by even high concentrations of cyanide, whereas the more purified compound loses its light-producing characteristics in the presence of extremely low cyanide concentrations. (Giese and Chase, 1940.)

The luciferase used with this purified luciferin, when studying the luminescent reaction *in vitro,* is ordinarily prepared by dialyzing a water extract of powdered dry *Cypridina* against distilled water for some days at a low temperature until the solution has become practically colorless. Considerable inactive protein is precipitated by this treatment and most of the luciferin and other dialyzable compounds are certainly eliminated. That luciferin can pass through a dialysis membrane was demonstrated by Harvey (1917), and it seems reasonable to assume that oxidized luciferin is also dialyzable, although this has not actually been shown.

Anderson found (1936), with luciferin purified by his method (1935), that the luminescent reaction of luciferin and luciferase, which requires the presence of oxygen, was irreversible. On the other hand, he found that the non-luminescent oxidation of luciferin which occurs in the presence of dissolved oxygen or of certain oxidants (*e.g.,* ferricyanide) can be almost completely reversed by $Na_2S_2O_4$ or suitable reductants, if the latter are added soon enough. Anderson concluded that the luminescent reaction of luciferin and luciferase and the non-luminescent oxidation of luciferin are quite different from each other. He found a redox potential for the reversible oxidation of luciferin, assuming that the oxidation removed two electrons per molecule, about 0.01 volt negative to quinhydrone at 23° and pH 6.8 (Anderson, 1936). Korr (1936) found a similar value. E_0' is, then, about +0.260 volt at pH 7. Anderson pointed out the similarity of this value to the redox potentials encountered in the case of certain naturally occurring polyhydroxybenzene derivatives studied by Ball and Chen (1933). He has emphasized the possibility that such a grouping may be present as part of the luciferin molecule. It is interesting to note that, in the work of Ball and Chen, the oxidized forms of the natural compounds whose potentials they measured were very unstable. In fact, it was necessary to use a flow technique in order to make the measurements. This feature of these compounds again shows a similarity to the luciferin-oxidized luciferin system, where the oxidized luciferin also seems to be unstable, since it cannot be reduced if it has stood too long in the presence of an oxidizing agent or of dissolved oxygen.

Anderson (1936) studied the reversible non-luminescent oxidation of purified luciferin in some detail and found that the luminescent reaction which occurred upon adding luciferase to a solution of luciferin that had been exposed to air for a short time showed evidence of two distinct kinds of luminescence, a rapid and a slow luminescence, occurring simultaneously. He attributed the intense, rapid luminescence to reduced luciferin in the solution and the dim, slow luminescence to reversibly oxidized luciferin which was being reduced in the presence of luciferase or of compounds extracted with it.

The oxidation of luciferin can be accelerated by irradiation with certain parts of the spectrum, as was shown by Harvey (1925, 1926). He used crude luciferin extracts from *Cypridina* and found that blue, violet, and near ultraviolet light were all effective in accelerating the oxidation. Chase and Giese (1940) later found that, when purified luciferin was used, the ultraviolet below 3000 Å was effective, but ultraviolet of longer wavelength and the visible part of the spectrum were not, unless a photo-sensitizing compound was added. A number of compounds were found to act as sensitizers, including eosin and fluorescein. Boiled, crude aqueous extracts of *Cypridina,* containing oxidized luciferin and all the other water-soluble components of the organism, were very effective, indicating that impurities in the crude extract were sensitizing the oxidation of luciferin irradiated by visible light in the case of Harvey's earlier experiments.

Chase and Giese found that, when the luminescent reaction was measured upon adding luciferase to luciferin that had been irradiated with ultraviolet light or with visible light in the presence of a sensitizer, the reaction could be differentiated into two parts, one a bright luminescence and the other a dim luminescence, as Anderson had found in the case of luciferin that had stood in the presence of dissolved oxygen. An interesting exception occurred when the purified luciferin was irradiated with visible light in the presence of riboflavin as a sensitizer. In this case, the luminescent reaction which ensued on addition of luciferase lacked the dim component, as shown in FIGURE 1. Riboflavin evidently prevents the reduction by luciferase (or whatever is responsible) of reversibly-oxidized luciferin. However, riboflavin is effective in this way only if the oxidation of luciferin is caused by the action of light with the flavin as sensitizer. If the luciferin is oxidized by dissolved oxygen, in the dark, with riboflavin present in the solution, and luciferase is then added, the resulting luminescence shows the bright and dim component ordinarily observed.

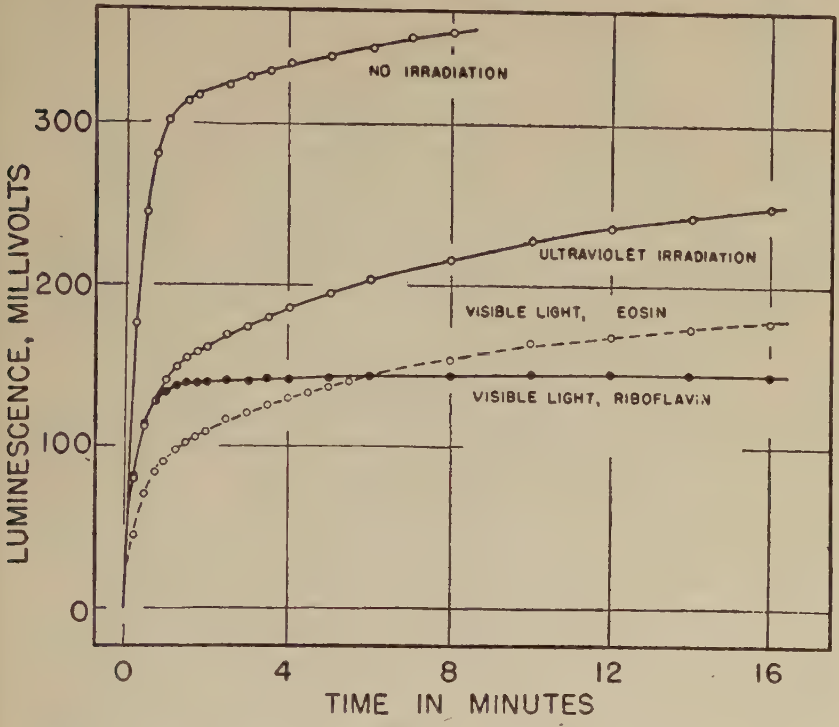

FIGURE 1. Typical luminescence curve obtained on adding luciferase to luciferin solutions which have been irradiated for three minutes with visible light in the presence of about 0.001 per cent concentration of riboflavin. Although an initial bright luminescence occurs, there is no light-emission after two minutes. The luminescence curves obtained after irradiation of luciferin with visible light with eosin present, and after ultraviolet irradiation without a sensitizer, both show not only a bright luminescence during the first two minutes, but also a dim luminescence which persists for at least sixteen minutes.

Recently, Chase and Lorenz (1945) separated the velocity constants of the enzyme-catalyzed luminescent reaction of luciferin and of its non-luminescent oxidation. They measured the luminescent reaction at five temperatures and fitted the data with an equation representing two first-order reactions occurring simultaneously. FIGURE 2 shows their experi-

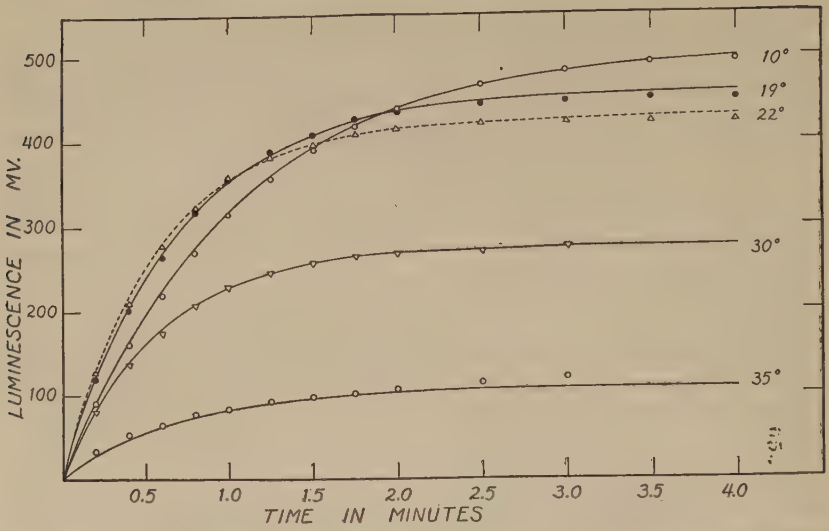

FIGURE 2. The luminescent reaction at five temperatures. The symbols represent experimentally measured total light emitted from the moment of mixing luciferin and luciferase to various times. The curves are theoretical, calculated from an equation derived on the assumption that two first order reactions are occurring simultaneously: one, the luminescent reaction of luciferin, and the other, its non-luminescent oxidation.

mental points and the curves which describe them are theoretical. Since Anderson (1936) has demonstrated that the luminescent reaction of luciferin and luciferase is a different process from the non-luminescent oxidation of luciferin, it is clear that both these processes may occur simultaneously. Chase and Lorenz assumed that, since dissolved oxygen is probably present in excess, the non-luminescent oxidation of luciferin might obey a first-order equation even though it might actually be a bimolecular reaction. Amberson had shown (1922) that the luminescent reaction obtained on mixing crude *Cypridina* luciferin and luciferase extracts obeyed the equation for a first order reaction. A record of the logarithmic decay of *Cypridina* luminescence is shown in the paper by Harvey (1948). The equation which describes the curves of FIGURE 1 was derived by Chase and Lorenz on the assumption, then, that the luciferin is the principal substrate in two first-order reactions occurring simultaneously, only one of which results in light emission. If x is the luciferin consumed by the luminescent reaction after time, t, and y is the luciferin consumed by the non-luminescent oxidation reaction after time, t, and if k_1 and k_2 are the respective velocity constants and a is the concentration of luciferin initially present, then:

$$\left\{\begin{aligned}\frac{dx}{dt} &= k_1\,(a - x - y)\\ \frac{dy}{dt} &= k_2\,(a - x - y)\end{aligned}\right\}\text{are to hold simultaneously.}$$

This gives $\frac{dy}{k_2} = \frac{dx}{k_1}$, whence, since $y = 0$ when $x = 0$, $y = \frac{k_2}{k_1}x$.

Using this value of y, $\frac{dx}{dt} = k_1\left(a - x - \frac{k_2}{k_1}x\right)$.

On integration and simplification, one obtains

$$x = \frac{k_1 a}{k_1 + k_2}\left(1 - e^{-(k_1 + k_2)t}\right)$$

This equation describes the data of FIGURE 2 rather exactly, the values of k_1 and k_2 being obtained directly from the experimental measurements.

If the logarithm of k_2 (the velocity constant of the non-luminescent oxidation reaction) be plotted against the reciprocal of the absolute temperature, a fairly straight line is obtained, as shown in FIGURE 3, and the slope of this line corresponds to an energy of activation of about 25,000 calories per mole. The velocity constant of the luminescent reaction, on the other hand, has a maximum value at about 23° C, the principal effect of temperature in this case being upon the enzyme, luciferase.

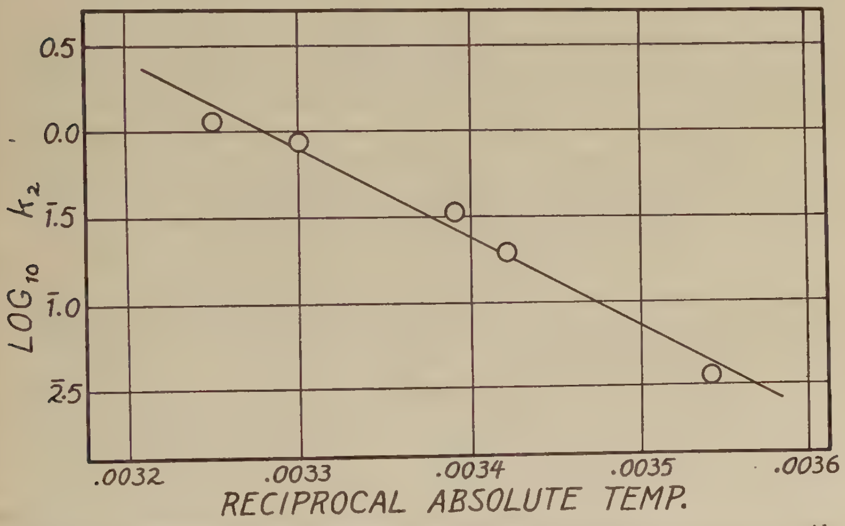

FIGURE 3. Logarithm$_{10}$ of the calculated velocity constant of the non-luminescent oxidation of luciferin plotted against the reciprocal of the absolute temperature. The slope represents a value of about 25,000 calories for the energy of activation.

Although the subject of this paper is the chemistry of luciferin, rather than the kinetics of the luminescent reaction, it seems proper to include one more datum from the kinetic point of view, in addition to those already mentioned.

Chance, Harvey, Johnson, and Millikan (1940) measured, by means of a special technique, the luminescent reaction of unpurified *Cypridina* luciferin and luciferase mixed in two different ways. In the first method, luciferin, in solution containing dissolved oxygen, was mixed with luciferase, also in solution containing oxygen. In the second method, a de-aerated solution containing both luciferin and luciferase was mixed with a solution containing dissolved oxygen. The luminescent reaction was much faster when the luciferase and luciferin were previously mixed than it was in the other case. The data were interpreted as showing that a combination between luciferin and luciferase is an essential requirement for luminescence, and that this combination represents a relatively slow reaction. The analysis also showed that the enzyme, luciferase, is the light-emitting molecule. This latter interpretation has generally been made, although it has not been proved that the light-emitting molecule may not be luciferin.

So far as the chemical structure of *Cypridina* luciferin is concerned, very little is known with certainty. However, several hypotheses have been advanced, most of them backed by some experimental evidence. The method of purification rules out any possibility that luciferin is a protein, while the redox potential measurements mentioned above have shown a possible relationship to hydroxybenzene derivatives. Anderson (1936) also interpreted the formation of benzoyl derivatives of luciferin as indicating the presence of reactive hydrogen which might be attached to nitrogen, sulfur or oxygen. Chakravorty and Ballentine (1941), however, found no nitrogen or sulfur in luciferin.

Giese and Chase (1940) found that luciferin purified by Anderson's method, irreversibly lost its property of giving luminescence with luciferase when very small concentrations of cyanide were present, as illustrated in FIGURE 4. On the other hand, luciferin in crude extracts of *Cypridina* is not affected by high cyanide concentrations. This indicated a chemical reaction between luciferin and cyanide, and Giese and Chase calculated a molecular weight for luciferin of between 800 and 2400, assuming the luciferin to be 100 per cent pure and assuming a 1:1 combination between the luciferin and the cyanide. Since the luciferin is certainly not pure, the true value for the combining weight is probably at least as low as 800 or even less. Giese and Chase interpreted the reaction with cyanide as due to cyanhydrin formation, and assumed an aldehyde or keto group on the molecule, which group might also be the site of combination with luciferase in the irreversible luminescent reaction.

With a view toward obtaining corroborative data on the combining weight of luciferin, an attempt was made recently, in collaboration with Dr. C. B. Anfinsen (unpublished), to determine an oxygen uptake by purified luciferin during its non-luminescent and luminescent oxidations, using the Cartesian diver method, and assuming two atoms of oxygen

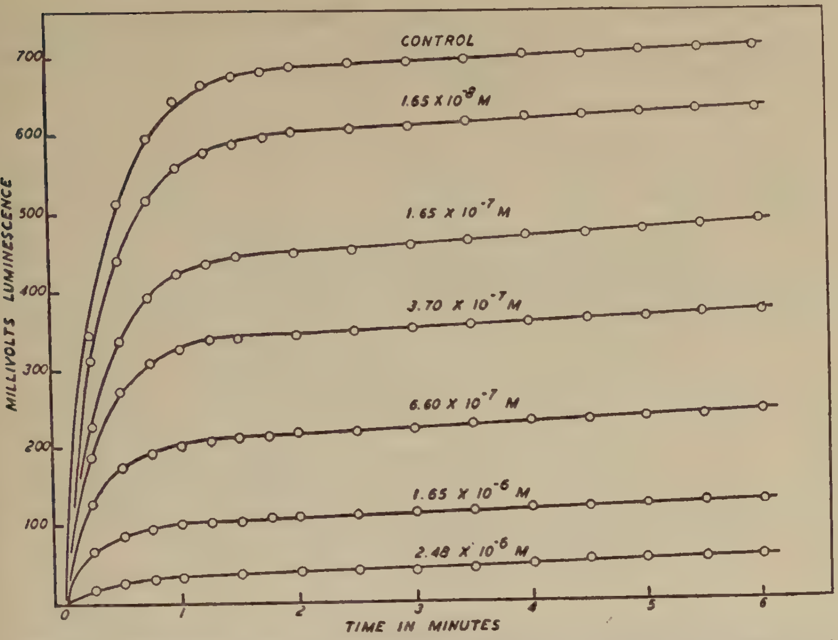

FIGURE 4. Quantity and rate of light-emission from identical samples of luciferin which were treated with various concentrations of cyanide, indicated on the curves, before addition of luciferase. Since the velocity constants of the curves are essentially the same, the luciferin rather than the luciferase is affected by the cyanide.

combined per molecule of luciferin in the non-luminescent oxidation. Although there were indications of oxygen uptake in some cases, the results were inconclusive. Parallel tests on the stability against oxidation of comparably small samples of luciferin solution, made it appear likely that the combination with oxygen, assuming that it does occur, took place during the time the divers were being filled and before the actual measurements of gas uptake could be started. R. S. Anderson has unpublished data on the concentration of ferricyanide necessary to oxidize known amounts of luciferin, which indicate a combining weight of about the same order of magnitude found in the case of the cyanide experiments (personal communication). The uncertainty in all measurements of the combining weight of luciferin by such methods lies chiefly in the assumption as to the purity of the luciferin. The cyanide data of Giese and Chase certainly point to a combining weight (and probably molecular weight) of less than 1000, perhaps about half this value. There seems little doubt that luciferin is a relatively small molecule.

Chase found (1942) that the luminescent reaction of purified luciferin and luciferase was reversibly inhibited by sodium azide. Since the total light was affected but not the velocity constant, the action of the azide was attributed to a reversible combination with the luciferin rather than with the luciferase. As was pointed out in another connection by Johnson, Eyring, and Williams (1942; see page 259), this interpretation may not be justified in the case of a reversible combination. The reaction might equally well take place between the azide and luciferase, although this does not seem likely. FIGURE 5A shows the effect of azide concentration upon the total light emitted. Experiments were run at pH 5.4 and at pH 6.6 and, as the figure shows, the azide was more effective at the lower pH. Since the pK of hydrazoic acid is about 4.7, a higher concentration of the undissociated acid would be present at pH 5.4 than at pH 6.6. For this reason, the effect was attributed to the undissociated acid rather than the azide anion.

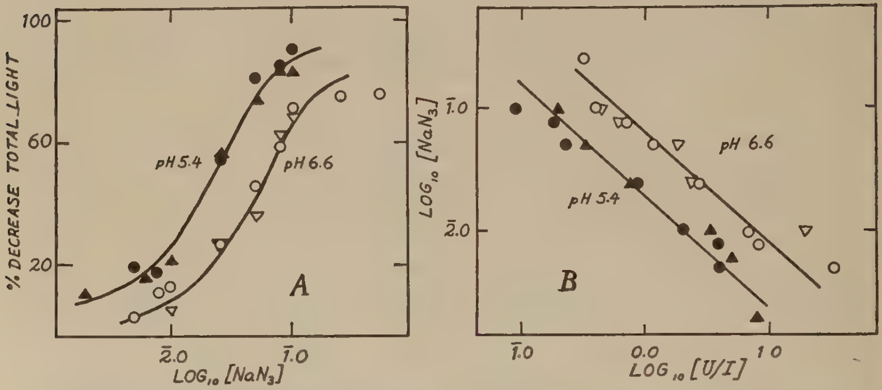

FIGURE 5. A. Per cent decrease of total light plotted against the logarithm of the sodium azide concentration. Four series of experiments are shown, two at pH 6.6 and two at pH 5.4. A given azide concentration is more effective at the lower pH, indicating that HN_3 may be involved.

B. The same data plotted in terms of a mass law equation. The slopes of the lines are approximately unity, which may indicate that one azide molecule or ion combines with a single luciferin molecule.

Since this reaction with azide is reversible, the data can be put into a form that makes them analyzable in terms of a mass law equation, as has been done in cases of inhibitor action on enzyme systems (see, for example, Fisher and Öhnell, 1940). In the present case, the analysis is as follows:

$$\text{Luciferin} + a \text{ azide} \rightleftarrows \text{luciferin (azide)}_a$$

$$\frac{[\text{Luciferin}]\,[\text{Azide}]^a}{[\text{Luciferin (Azide)}_a]} = K$$

$$\frac{[\text{Luciferin}]}{[\text{Luciferin (Azide)}_a]} = K \cdot [\text{Azide}]^{-a}$$

If [*Luciferin*] is called U and is put equal to the percentage of luminescence that occurs at any given azide concentration, taking as 100 per cent the luminescence obtained when no azide is present, and if [*Luciferin (Azide)$_a$*] is called I and is put equal to the percentage of potential luminescence that does not occur, then,

$$\frac{U}{I} = K \bullet [\text{Azide}]^{-a}, \text{ or } \log\frac{U}{I} = -a \log[\text{Azide}] + \log K.$$

Therefore, by plotting $\log_{10}\frac{U}{I}$ against the $\log_{10}$ of the azide concentration, a straight line should be obtained and the slope of the line should give the value of a, the number of azide molecules which combine with a single luciferin molecule. FIGURE 5B shows the data plotted this way. It is apparent that the slope is about –1, indicating one molecule of hydrazoic acid combining reversibly with each molecule of luciferin.

Fieser and Hartwell (1935) studied the reaction of hydrazoic acid with benzo- and naphthoquinones. According to them, an azidohydroquinone is first formed, changing to an amino-quinone with liberation of nitrogen. Although a reaction of this sort would hardly be considered as readily reversible, it seems possible that a related type of reaction might occur in the case of luciferin and, if so, would be further evidence for a quinonoid structure.

Chase has shown (1945) that luciferin, freshly dissolved in pH 6.8 phosphate buffer in absence of air, has an absorption band at about 435 mμ in the visible spectrum. Measurements of the absorption spectrum were made with a recording spectrophotometer (Hardy, 1935). If dissolved oxygen is present, this band is rapidly replaced by another at about 465 mμ and this latter band then disappears slowly, leaving a practically colorless solution (Chase, 1943). These changes in the visible absorption spectrum of luciferin solutions take place much more slowly if the pH is more acid than 6.8. For example, as FIGURE 6 illustrates, at pH 5.1 the change is only about one-fifth as fast. Furthermore, at pH 5.1 there is definite evidence, from the absorption spectrum, for the production of a compound having acid-base indicator properties during the exposure of the luciferin to dissolved oxygen. This compound absorbs more strongly in the violet and near ultraviolet at a pH of 5.1 than it does at pH 6.8, as is also shown in the curves of FIGURE 6. The rate of change of the absorption spectrum increases with increase of pH in about the same way that the rate of non-luminescent oxidation of luciferin varies with pH in solutions containing dissolved oxygen (Chase, 1940). In fact, there is little doubt (Chase, 1943) that the amount of labile color in a luciferin solution is directly proportional to the concentration of luciferin, as measured by the total light obtainable from the solution.

If the visible absorption spectrum of a luciferin solution is measured during the luminescent reaction of luciferin and luciferase (as can be done with the Hardy recording spectrophotometer, which is not affected

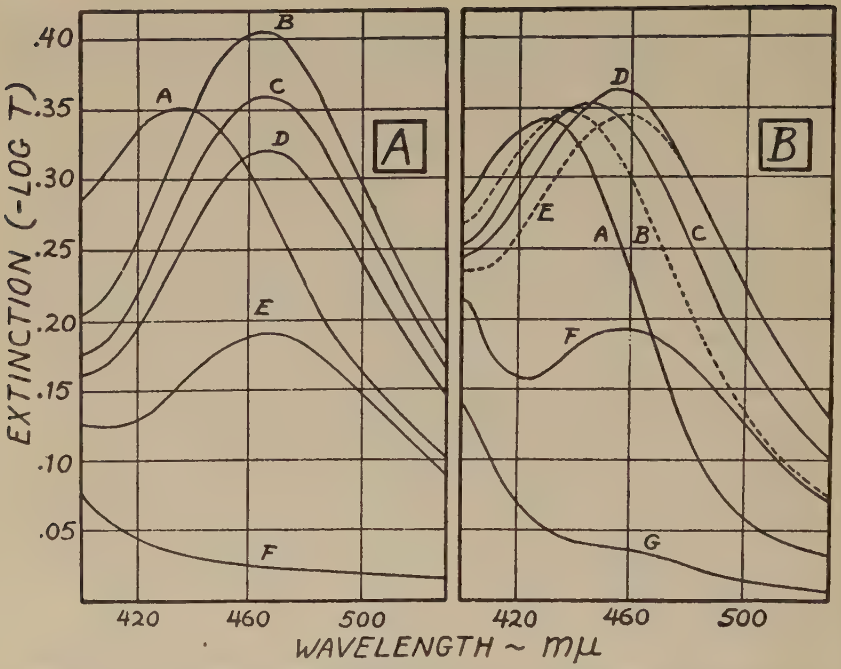

FIGURE 6. The left half shows the changes which occur in the visible absorption spectrum of a luciferin solution at pH 6.8 during exposure to air. Curve A is the spectrum 5 minutes after the luciferin was dissolved. Curve B was measured after a total elapsed time of 19 minutes, curve C after 31 minutes, curve D after 44 minutes, curve E after 81 minutes, and curve F after 2 days.

The right part of the figure shows similar measurements on a luciferin solution at pH 5.1. Curve A was measured 4 minutes after the luciferin was dissolved, curve B after 16 minutes, C after 30 minutes, D after 59 minutes, E after 81 minutes, F after 300 minutes, and G after 26 hours.

by continuous light-emission in the sample), it is found that the same changes take place, but that they occur one hundred times as fast as during the spontaneous, non-luminescent oxidation of luciferin in absence of the enzyme. Absorption spectra measured during the luminescent reaction and during the non-luminescent oxidation of luciferin are compared in FIGURE 7.

It seems possible that the shift of absorption maximum from 435 mμ to 465 mμ may be correlated with the reversible oxidation of luciferin demonstrated by Anderson (1936). The two facts that, first, the products of the luminescent reaction of luciferin and of its reversible, non-luminescent oxidation are evidently different (Anderson, 1936) and that, second, the changes in the absorption spectrum are the same during the two reactions (Chase, 1943), would not appear to support such an interpretation. However, the two reactions might be different without necessarily causing a difference in the light-absorbing groups of the molecule. Further experimental evidence is needed before more than tentative conclusions can be drawn. If the assumption be granted that the shift in spectral absorption actually represents the reversible oxidation, then this might indicate the oxidation of a polyhydroxybenzene derivative or related compound. So far, no attempt has been made to demonstrate the reverse shift of the absorption band from 465 mμ to 435 mμ as a result of reduction of spontaneously oxidized luciferin.

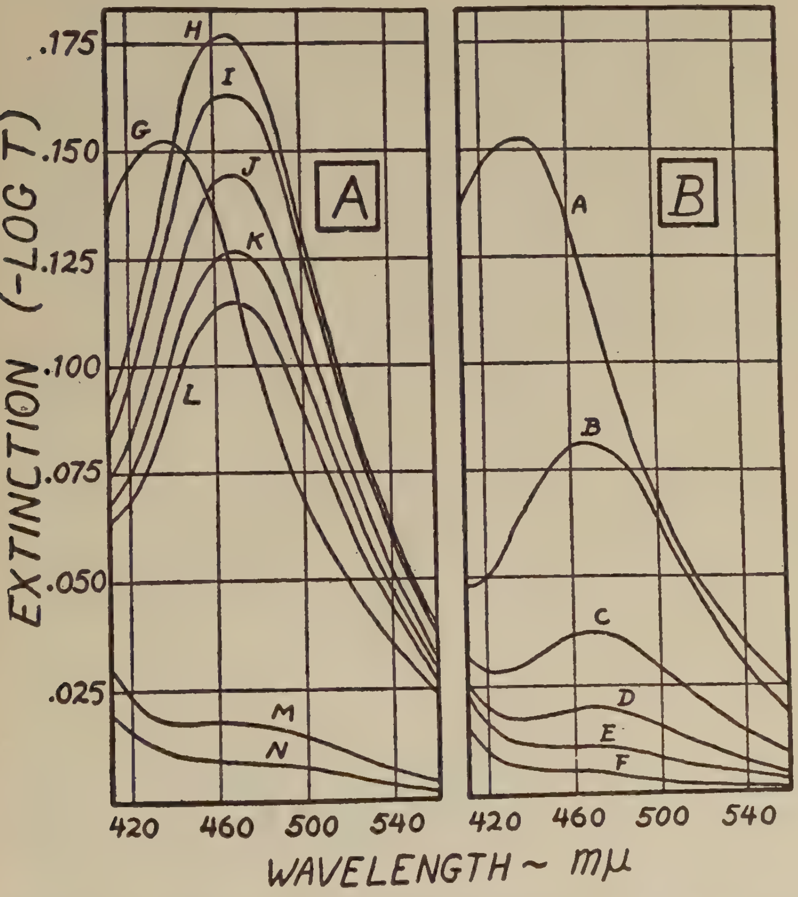

FIGURE 7. The curves shown in the left half of the figure are absorption spectra of a luciferin solution measured during exposure to air. Curve G is the spectrum immediately after the luciferin was dissolved; H, I, J, K, L, M and N, after 9, 20, 30, 39, 49, 455 and 1140 minutes, respectively.

In the right half of the figure are shown absorption spectra of a luciferin solution in which luminescence is occurring due to the action of luciferase, the enzyme. Curve A is the spectrum immediately after the luciferin was dissolved, but before the luciferase was added. Curve B was measured 1 minute after the luciferase was added, curve C after 12 minutes, D after 22 minutes, E after 40 minutes and F after 457 minutes. The changes in the absorption spectrum are about 100 times as rapid during luminescent oxidation by luciferase as during non-luminescent oxidation.

The slow disappearance of the 465 mμ band upon prolonged exposure to dissolved oxygen, or its rapid disappearance in the presence of luciferase, may indicate the disruption of a ring structure. If this be true, it is not surprising that the luminescent oxidation of luciferin should be irreversible. As mentioned earlier, the oxidation products of the naturally occurring polyhydroxybenzene derivatives studied by Ball and Chen (1933) were also unstable compounds. It has been shown by Hooker (1936) that during oxidation by alkaline potassium permanganate, certain 2-hydroxy-1,4-naphthoquinone derivatives undergo an opening of the quinone ring followed by subsequent closure. If a similar reaction occurred in the irreversible oxidation of luciferin (although there is, of course, no experimental evidence for such a mechanism), it seems quite conceivable that the ring, having opened, might become so constituted that it could not close. This would be a possible explanation for the irreversible step in the oxidation, the reversible step having been the oxidation to a quinone.

Chakravorty and Ballentine (1941), in a short note, summarized the then available chemical data on *Cypridina* luciferin and suggested a partial structure for the molecule, illustrated in FIGURE 8. The authors found further evidence for a keto group from the fact that a microcrystalline precipitate was obtained upon treatment of luciferin with hydroxylamine acetate. They suggested that the irreversible luminescent reaction involved the oxidation of the side chain to a carboxyl group, as follows:

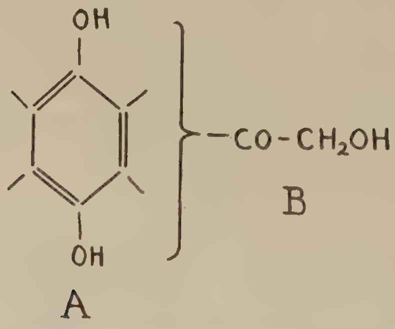

FIGURE 8. The partial structure suggested for luciferin by Chakravorty and Ballentine (1941). The group labeled "A" would represent the part of the molecule capable of reversible oxidation and reduction but not necessarily concerned in the luminescent reaction. The side chain labelled "B" would be capable of reacting with benzoyl chloride or cyanide or, perhaps, with luciferase, and could be oxidized to a carboxyl group as a result of the luminescent reaction with the enzyme.

$$\text{R-CO-CH}_2\text{OH} \xrightarrow[(\text{O}_2)]{\text{luciferase}} \text{R-COOH.}$$

If this were correct, they reasoned that it should be possible to regenerate the starting material from the product of the luminescent reaction by appropriate chemical treatment, thus:

$$\text{R-COOH} \xrightarrow{SOCl_2} \text{R-COCl} \xrightarrow{CH_2N_2} \text{R-CO-CHN}_2$$

$$\text{R-CO-CHN}_2 \xrightarrow{\text{dil. } H_2SO_4} \text{R-CO-CH}_2\text{OH.}$$

After having treated luciferase-oxidized luciferin in this way, they obtained a compound which, they believed, gave luminescence with luciferase, greater in intensity than was obtained from their control. Unfortunately, no quantitative measurement of the luminescence was made, and the light which was obtained was probably only a very small percentage of that which should have been expected, considering the high concentration of luciferase-oxidized luciferin in their starting material. The experiment is especially significant, however, in that it represents probably the first attack upon the problem of the structure of luciferin by the methods of organic chemistry. A continuation of this kind of approach should prove fruitful. Chakravorty and Ballentine also reported microchemical analyses of samples of purified luciferin, which showed no nitrogen, sulfur, halogen, or ash.

McElroy and Ballentine (1944) found evidence for the presence of acid-labile phosphate in purified luciferin. They also reported a significant increase in inorganic phosphate during the luminescent reaction, and stressed the possibility that formation of an energy-rich phosphate bond may be involved in the luminescent reaction. Eymers and van Schouwenburg (1937) had measured the emission spectrum of crude extracts of *Cypridina* luciferin and luciferase. Their analysis showed two fundamental frequencies: one of 21,250, corresponding to a wavelength of 470 mμ, and another smaller component of 18,200, corresponding to a wavelength of 549 mμ. These frequencies represent energies of about 59,000 and 50,000 calories, respectively, and, according to McElroy's and Ballentine's calculations, the preservation of energy as phosphate bond energy from oxidation of the postulated side chain of luciferin, and the subsequent release of this energy through reaction with luciferase would be sufficient to produce luminescence having the observed spectral quality.

If luciferin does have an hydroxybenzene, or related, structure and if it can be reversibly oxidized to a quinone, measurements of its ultraviolet absorption spectrum would be expected to throw light on the specific configuration of the molecule. The only such measurements so far recorded (Chase, 1943; Chase and Giese, 1940) cannot be regarded too seriously, since impurities were certainly present in the luciferin solutions even after two cycles of purification by Anderson's procedure (1935). Impurities would doubtless cause much greater interference with the ultraviolet spectrum than in the visible. An ultraviolet and visible absorption spectrum of luciferin in pH 6.8 phosphate buffer is shown in FIGURE 9, measured immediately after dissolving the luciferin and after exposure to air during two time-intervals. A qualitative resemblance to the spectra of *p*-quinone and of certain naphtho- and anthraquinone derivatives actually seems to exist, and it is possible that luciferin may have an aromatic ring structure as its nucleus. Since the solvent frequently exerts a considerable effect on the absorption spectrum of a solution, precise measurements of the absorption spectrum of luciferin should be made, using a variety of solvents, in order to secure data susceptible of interpretation in terms of structure.

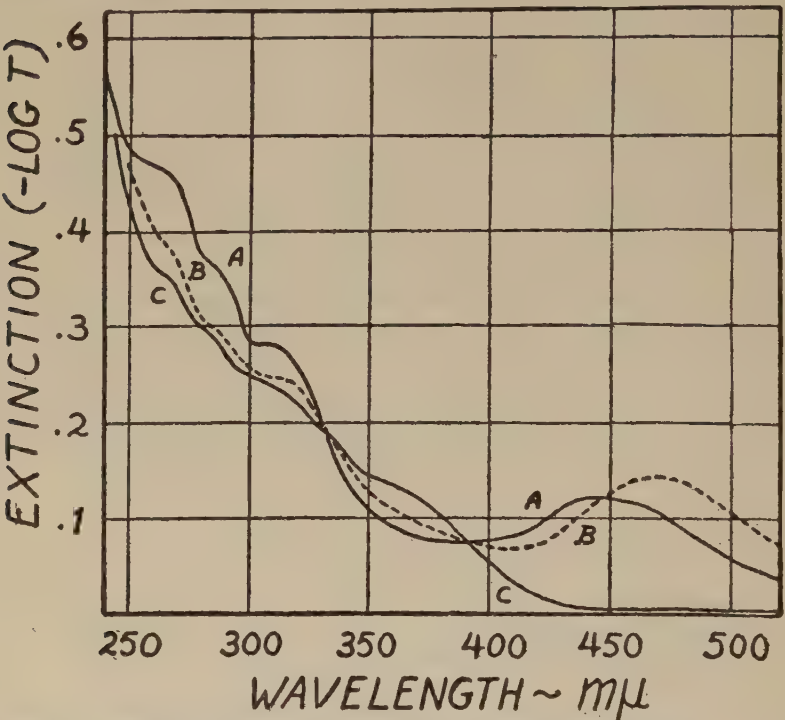

FIGURE 9. Visible and ultraviolet absorption spectra of a luciferin solution at pH 6.8. The spectra were recorded with the Harrison and Bentley spectrophotometer (1940). Curve A was measured as soon as possible after the luciferin had been dissolved. Curve B was measured after exposure of the solution to air for 32 minutes, and curve C after 24 hours.

With the ultraviolet absorption spectrum measurements, such as they are, favoring *p*-quinone, naphthoquinone or anthraquinone derivatives, the first-mentioned structure seems most acceptable in view of the fact that the E_0' (pH 7) of the luciferin system is almost identical with that of quinhydrone (Anderson, 1936; Korr, 1936) and not sufficiently negative to fall in the range characteristic of naphtho- and anthraquinones. It must be remembered, however, that substitution in a quinone ring can greatly change the oxidation-reduction potential in either a positive or negative direction, depending upon the nature of the substituent group.

As stated earlier, luciferin and luciferase have not yet been extracted from luminous bacteria although there is every reason to believe that they occur. Recently Kluyver, van der Kerk, and van der Burg (1942) determined the action spectrum for the inhibition of bacterial luminescence by light of various wavelengths in the visible and ultraviolet. Assuming that this action spectrum represented the absorption spectrum of a compound closely related to bacterial luciferin, probably oxidized luciferin, they tentatively identified this compound from its spectrum, and from other considerations, as a 1,4-naphthoquinone derivative with a ketohydroxy group directly substituted in the quinone ring. They based their interpretation largely upon the conclusions of Chakravorty and Ballentine (1941), discussed above. There is a possibility, as the authors point out, that the action spectrum in this case may be affected by inert colored compounds in the medium or in the bacterial cell, or that it may represent the absorption spectrum of some compound which is so acting as to sensitize to light a component of the luminescent reaction. The action spectrum as measured might not, therefore, necessarily represent the absorption spectrum of oxidized luciferin. Although the actual comparison of the measured action spectrum with the absorption spectrum of the naphthoquinone derivative is not shown in their paper, it does appear in the doctorate thesis of van der Kerk (1942). While there is a qualitative resemblance between the two spectra as far as shape is concerned, the actual positions of absorption bands differ rather widely. Furthermore, the spectrum of the naphthoquinone derivative was measured in hexane, whereas the solvent for the hypothetical oxidized bacterial luciferin is unknown but is presumably largely aqueous. For these reasons, the conclusions as to structure should, as the authors themselves stress, be considered as purely tentative.

Another investigation on the luminescent reaction in bacteria which has yielded information as to certain characteristics of bacterial luciferin is that of Johnson, van Schouwenburg, and van der Burg (1939). They recorded the flash of luminescence in bacteria, following anaerobiosis under a variety of conditions. They concluded* that, since their data indicated that the luminescent oxidation of luciferin by luciferase and oxygen is evidently irreversible, whereas the dark oxidation by a substrate (or its breakdown product) is reversible, the situation is rather closely comparable to that in *Cypridina* extracts. Anderson (1936) had shown that the luminescent and non-luminescent oxidations of *Cypridina* luciferin resulted in different products. Johnson, van Schouwenburg, and van der Burg suggested, in addition, that the two oxidations might well occur on different portions of the luciferin molecule. That is, that the molecule might have one group which might easily undergo reversible oxidation and reduction, while an entirely different group might be irreversibly oxidized with light emission.

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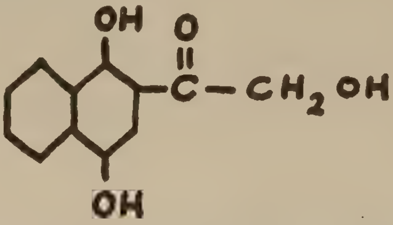
* See page 211 of their paper.

A privately printed doctoral thesis by C. J. P. Spruit (1946) has recently appeared. This contains, among other things, the ultraviolet absorption spectra of a number of compounds with structures related to those that have been suggested for luciferin as a result of the work of Anderson (1936), Giese and Chase (1940), Chakravorty, and Ballentine (1941), van der Kerk (1942), Kluyver, van der Kerk, and van der Burg (1942), and Chase (1943). Spruit concludes that bacterial luciferin may have the following structure:



The compounds whose absorption spectra he measured include some thirty-three anthra-, naphtho- and benzoquinone and hydroquinone derivatives. Since Spruit's conclusion as to the structure of bacterial luciferin depends upon a comparison of the spectra of these known compounds with that of luciferin, the absorption spectrum of luciferin itself should be established more definitely than has so far been possible before the structure suggested by Spruit can be considered proven. The ultraviolet absorption spectra so far reported for *Cypridina* and bacterial luciferin, whether measured by direct or indirect methods, may easily have been distorted by the presence of impurities. Consequently, comparisons with the spectra of known compounds must be made with caution and a good deal of reservation.

Johnson (1947) in a comprehensive review paper on bacterial luminescence and Johnson and Eyring (1944) have called attention to the possibility that luciferin may be a prosthetic group on the protein enzyme, luciferase. Part of their evidence for this hypothesis is their demonstration that *Cypridina* luciferase solutions which have been subjected to prolonged dialysis can be made to luminesce by the addition of $Na_2S_2O_4$, followed by aeration of the solutions. They suggest that this prosthetic group may possibly be of flavin nature, although most of the experimental evidence available at present would appear not to favor such a structure for the luciferin molecule (Anderson and Chase, 1944; McElroy and Ballentine, 1944).

The purification of *Cypridina* luciferin by crystallization has been a goal of all workers in the chemical field of bioluminescence since luciferin was first extracted but, save for one doubtful preliminary report, no one has succeeded in obtaining crystals. Kanda (1932) reported crystallization of luciferin but his paper did not contain photographs of the crystals. The work has not been confirmed by other investigators nor have further papers on the subject been published by him.

The only crystalline derivative of luciferin so far reported is the microcrystalline precipitate mentioned by Chakravorty and Ballentine (1941), which was obtained on treatment with hydroxylamine acetate. Here, too, no photographs are shown. When the irreversible reaction between purified luciferin and cyanide (Giese and Chase, 1940) was first observed, indicating the possible presence in luciferin of an aldehyde or keto group, an attempt was made to obtain a crystalline derivative by reaction with 2,4-dinitrophenylhydrazine. However, this was not successful. Obviously, the formation of any kind of crystalline derivative, whether capable of producing light with luciferase or not, would be valuable as a means of obtaining material of a high degree of purity for quantitative analysis, as a lead towards establishment of structure. The scarcity of the starting material, *Cypridina,* means that micro-methods, rather than the easier macro-techniques, must be used in all such work.

It may be interesting to summarize briefly some unpublished data by Dr. Harvey and myself on relative solubilities of *Cypridina* luciferin, both crude and purified, in various solvents. The amount of luciferin in solution was determined by measuring the total light available from an aliquot of the solution, on addition of luciferase, at constant pH. As had been observed earlier by Harvey, by Kanda, and by Anderson, we, too, found that great differences in the relative solubility in different solvents existed, depending upon whether the crude, dry *Cypridina* powder or the purified luciferin were used. The purified luciferin (Anderson's method), in the quantity used, was found to be completely soluble in the following solvents: methanol, ethanol, butyl alcohol, isobutyl alcohol, and propyl alcohol. Amyl alcohol, acetone, chloroform, aniline, and 0.06 *M*, pH 6.8 phosphate buffer dissolved only 50-75 per cent of the available luciferin. Ethyl ether, petroleum ether, and benzene dissolved none.

Luciferin in the form of the dry, powdered *Cypridina* material was found to be most soluble in methanol. Calling this value 100 per cent, ethanol dissolved about 16 per cent, propyl alcohol about 10 per cent, and butyl alcohol, isobutyl alcohol, amyl alcohol, acetone, chloroform, and ethyl ether dissolved practically none (0.5 per cent). Preliminary benzene extraction of the crude, powdered material in a Sohxlet caused a significant increase in the relative amounts of luciferin extractable by ethanol, butyl alcohol, and propyl alcohol. No measurable luciferin was removed by the benzene in the preliminary treatment. The details are given in TABLE 1.

TABLE 1

RELATIVE SOLUBILITIES IN VARIOUS SOLVENTS OF PURIFIED LUCIFERIN, LUCIFERIN IN DRY *Cypridina* POWDER, AND LUCIFERIN IN BENZENE-EXTRACTED DRY *Cypridina* POWDER

Solvent	Relative amount of luciferin extracted		
	Purified luciferin	Untreated *Cypridina* powder	Benzene-extracted powder
Methyl alcohol	98	100	100
Ethyl alcohol	94	16	34
Butyl alcohol	100	4	12
Isobutyl alcohol	100	5	—
Propyl alcohol	98	10	24
Amyl alcohol	83	0	5
Acetone	74	1	0
Chloroform	44	3*	6*
Ethyl ether	0	0	2
Petroleum ether	0	—	—
Benzene	0	—	—
Aniline	77	—	—
Phos. buff., pH 6.8	49	—	—

* Solid fragments present in the solution.

It seems quite clear, from these results on relative solubilities, that the luciferin, as it occurs in the crude state in the gland of *Cypridina,* must be bound in some way, or else be actually different, chemically, than it is in the purified condition. This same conclusion was reached earlier by both Harvey and Kanda. If there is an actual chemical difference, this cannot be great enough to alter the structure sufficiently to preclude reaction with the enzyme, luciferase. These differences between the unpurified and purified material certainly make it evident that experimental work must be confined to only one kind of luciferin preparation: preferably, at least at this stage, to luciferin purified by Anderson's method. Otherwise, inconsistencies are bound to occur which will confuse, rather than help to clarify, the problem.

As will have become apparent, the chemical structure of the luciferin of *Cypridina* is still far from known, although a number of facts have accumulated that bear directly on the problem, and several partial structures have been postulated on the bases of these data. It seems likely that the reversible oxidation may represent a reaction analogous to the oxidation of hydroquinone. Furthermore, the redox potential of the luciferin system certainly resembles those of naturally occurring polyhydroxybenzene derivatives whose oxidized forms are unstable, as is also the case with luciferin. The irreversible reaction between purified luciferin and cyanide may indicate a free aldehyde or keto group, and the chemical experiments of Chakravorty and Ballentine point to some sort of ketohydroxy side chain. The reaction with azide may indicate a quinone nucleus for reversibly oxidized luciferin, although this interpretation is only one of several which could be made in the light of those data. The evidence for a naphthohydroquinone structure with a ketohydroxy side chain for bacterial luciferin is probably not sufficiently conclusive to do more than indicate such a possibility. The published measurements of the ultraviolet absorption spectrum of *Cypridina* luciferin, although they, too, suggest an aromatic ring structure as the skeleton of the molecule, suffer from impurities and require remeasurement under a variety of conditions. The splitting-off of phosphate in the luminescent reaction certainly seems relevant in a consideration of structure.

A promising lead for determining the structure of luciferin may be its rather peculiar color change during oxidation. The change in spectral absorption from 435 mμ to 465 mμ on exposure to air and the subsequent loss of all visible light absorption on further exposure are rather unique properties. Any known compound which possesses such properties should be capable of transformation, by suitable chemical treatment, into luciferin. Since the luminescent reaction itself is enzyme-catalyzed, it is obvious that, in addition to the fundamental structure of the molecule, there must be one or more very specific groupings. The problem, then, once the basic structure has been found from absorption spectrum data, would involve making various derivatives by substitution of side chains, until a compound is obtained which can react with luciferase to give luminescence.

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THE FUNDAMENTAL ACTION OF PRESSURE, TEMPERATURE, AND DRUGS ON ENZYMES, AS REVEALED BY BACTERIAL LUMINESCENCE

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With any given enzyme reaction or more complex biological process, a full interpretation of the kinetics involves an understanding of the mechanisms through which temperature, hydrostatic pressure, and inhibitors of various kinds influence the observed rate. While the theory of equilibria in regard to temperature, pressure, and concentration of reactants has been known for some years, it is scarcely a decade since the rational and precise theory for change became available, in the theory of absolute reaction rates[1, 2] for a rate process such as that of a simple chemical reaction. Inasmuch as the same theory undoubtedly holds as well for each of the individual reactions which collectively lead to any biological phenomenon, the problem is to see how it may be applied to single reactions in the midst of many, and to what extent it may account, quantitatively, for the process as a whole. In some cases, for example with an extracted and purified enzyme system, the kinetics might be expected to be relatively uncomplicated, although simultaneous reactions involving the same molecule may influence the over-all rate of the measured process. In living cells, on the other hand, a series of systems is generally concerned, and under various conditions one or more systems may be largely rate-determining, in each case influenced in their activity by simultaneous equilibria and rate processes. When a single system remains largely limiting, however, it is reasonable to believe that even complex processes might be analyzed, approximately, in the manner of a single reaction.

For investigating the problem at hand, luminescence possesses distinct, and in certain respects unique advantages. In the first place, the intensity of the light is evidently proportional to the reaction velocity of the "luciferase" with "luciferin," as shown by kinetic studies with crude as well as with partially purified extracts of *Cypridina*.[3] Although luminescent extracts have not as yet been obtained from bacteria, spectroscopic and other lines of evidence indicate that the light-emitting system is not fundamentally different in the two cases. Inasmuch as the intensity of luminescence may be easily recorded by means of a photoelectric cell or other devices,[4] the rate of reaction, in relative units, can be determined with considerable accuracy for a given instant. Likewise, the course of more or less rapidly changing reaction rates may be followed during very brief as well as over longer periods of time.

In non-reproducing bacteria, under favorable physiological conditions, luminescence is in a steady state, with constant intensity. In extracts of *Cypridina,* the reaction is first order with respect to both the concentration of dihydroluciferin and of active luciferase, and the luminescent oxidation is accompanied by the destruction of a large part of the luciferin.[3, 5] In bacteria, if any considerable destruction of the luciferin accompanies the light-emitting oxidation, the luciferin must be formed from some precursor at the same rate, in order for a uniform intensity to be maintained. Since excited molecules are generally stabilized by radiating, whereas excited molecules which do not radiate are apt to be destroyed, repeated oxidation and reduction of the same luciferin molecules very likely occurs, with perhaps much less of the destructive reaction in living cells than in extracts. In any case, the uniform luminescence intensity of bacteria indicates that the concentration of the reactants, luciferin and luciferase, in effect remains constant with time. Thus, the intensity (I) of the light will be proportional to the amount of active luciferase (A_n) times the amount of reduced luciferin (LH_2) times the specific reaction rate constant, k, times some proportionality constant, b:

$$I = bk(A_n)(LH_2). \qquad (1)$$

While it is apparent that several equilibria and rate processes precede actual light emission[6] the evidence from flow-method studies with *Cypridina* extracts[7] indicates that EQUATION 1 represents the slowest reaction. A scheme of consecutive reactions consistent with the known facts concerning luminescence in bacteria and in extracts is given in TABLE 1. Excited luciferin is designated by L^*, and destroyed luciferin by L_1.

TABLE 1

Reactions with luciferase		Additional reactions that occur with and without luciferase	
)	$AL + XH_2 \rightleftharpoons ALH_2 + X$	(1′)	$L + XH_2 \rightleftharpoons LH_2 + X$
)	$A + LH_2 \rightleftharpoons ALH_2$		
)	$ALH_2 + O_2 \rightleftharpoons ALH + HO_2$	(3′)	$LH_2 + O_2 \rightleftharpoons LH + HO_2$
)	$ALH \rightleftharpoons AL^- + H^+$	(4′)	$LH \rightleftharpoons L^- + H^+$
) alpha	$AL^- + LH \rightarrow AL^* + LH^- \rightarrow AL + LH^- + h\nu$	(5′) alpha	$L^- + LH \rightarrow L^* + LH^- \rightarrow L + LH^- + h\nu$
) beta	$AL^- + LH \rightarrow AL + LH^-$	(5′) beta	$L^- + LH \rightarrow L + LH^-$
) gamma	$AL^- + LH \rightarrow AL_1 + LH^-$	(5′) gamma	$L^- + LH \rightarrow L_1$
)	$ALH + O_2 \rightarrow AL + HO_2$	(6′)	$LH + O_2 \rightarrow L + HO_2$
)	$AL + O_2 \rightarrow AL_1$	(7′)	$L + O_2 \rightarrow L_1$

In this scheme, the transfer of an electron between two semi-quinone forms of the luciferin leads to excitation, followed by radiation. The luciferin is presumed to be the radiating molecule, because of the correspondence between the absorption spectrum of luciferin and the emission spectrum of the luminescent reaction,[8, 9] together with the fact that luciferin emits light in 95 per cent alcohol at 70° C.,[10] a condition under which the enzyme might be expected to be inactive.

Thus, the over-all process of luminescence may be limited in various ways, even though reaction 2 ordinarily remains the slowest of the series. At very low oxygen tensions, for example, reaction 3 becomes limiting. In acid pH, the amount of ALH decreases and, to this extent, reaction 4 may be considered limiting. In alkaline pH, the amount of *LH* may be considered limiting, etc. All these reactions, however, are fast in comparison with reaction 2, even though alterations in the steady state concentration of subsequent reactants, *e.g.,* by changes in pH, influence the level of luminescence intensity in a corresponding manner. At optimum pH, with oxygen not limiting, and with excess of glucose, the over-all rate is determined by the specific reaction rate constant, the amount of luciferin, and the amount of active luciferase, as given in EQUATION 1.

A second, unusual advantage of luminescence in analyzing the kinetics of over-all processes that occur in living cells is that the enzyme system concerned is not preceded by a considerable number of other systems engaged in a stepwise hydrogen or electron transfer. The evidence that this is so derives from the facts that (a) glucose added to washed cells immediately results in large increases in luminescence, showing that some of the hydrogen from glucose is transferred *via* the luciferin-luciferase system, with light emission;[11, 12, 13] and (b) the wavelength of maximum intensity corresponds to a transition with $\Delta F^\circ = 60{,}700$ calories, as compared with the average ΔF° of 57,340 calories for two hydrogens in the oxidation of glucose.[6] Thus, in luminescence, some fraction of the hydrogens from glucose goes almost directly to oxygen, by way of the luminescent system, and the energy is released through visible radiation.

Finally, since luminescence may be studied both in living bacterial cells and in a corresponding system extracted and partially purified from *Cypridina,* it is possible to ascertain whether inhibitors which influence the intensity of the light in cells also act, at equivalent concentrations, on the system *in vitro.* Moreover, by measuring the effect of a given inhibitor on the total light in extracts, it is possible to distinguish between those which, like certain ions[14] quench luminescence, and those, such as urethane or sulfanilamide,[15] which retard the rate of the enzyme reaction without affecting the total light produced.

In order to account for the reversible effects of temperature and hydrostatic pressure on bacterial luminescence, it is necessary to modify EQUATION 1 to include an equilibrium between active and inactive forms of one of the reactants.[16, 17] Since this equilibrium, which rapidly becomes a conspicuous limiting factor with rise in temperature beyond the normal optimum, has been found to be characterized by the high heat and entropy typical of protein denaturation, it evidently concerns the enzyme.[18, 19, 20] Letting K_1 represent the equilibrium constant between active (A_n), and inactive or reversibly denatured (A_d) forms of the enzyme, and A_0 the total of $(A_n) + (A_d)$, Equation 1 becomes

$$I_1 = \frac{bk(LH_2)(A_0)}{1 + K_1}, \tag{2}$$

which, by definition of the equilibrium constant K_1 and of the rate constant k in accordance with the theory of Absolute Reaction Rates[1, 2] may be written:

$$I_1 = \frac{b\kappa \frac{kT}{h} e^{-\frac{\Delta F^\ddagger}{RT}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta F_1}{RT}}}$$

$$= \frac{b\kappa \frac{kT}{h} e^{-\frac{\Delta H^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta H_1}{RT}} e^{\frac{\Delta S_1}{R}}}$$

$$= \frac{b\kappa \frac{kT}{h} e^{-\frac{\Delta E^\ddagger}{RT}} e^{-p\frac{\Delta V^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta E_1}{RT}} e^{-p\frac{\Delta V_1}{RT}} e^{\frac{\Delta S_1}{R}}}. \tag{3}$$

Equation 3 contains some unknown quantities, (LH_2), (A_0), and $\Delta S^\ddagger$, which cannot be readily determined. Under chosen conditions, however, where luminescence of fully aerated, non-reproducing cells is constant with time, these quantities may be assumed essentially constant. The same applies to κ, the transmission coefficient, k, the Boltzman constant, h, Planck's constant, and b, the proportionality constant. Consequently, they may be included in a single constant, c, and Equation 3 then becomes

$$I_1 = \frac{cTe^{-\frac{\Delta H^\ddagger}{RT}}}{1 + e^{-\frac{\Delta H_1}{RT}} e^{\frac{\Delta S_1}{R}}} = \frac{c'Te^{-\frac{\Delta E^\ddagger}{RT}} e^{-p\frac{\Delta V^\ddagger}{RT}}}{1 + e^{-\frac{\Delta E_1}{RT}} e^{-p\frac{\Delta V_1}{RT}} e^{\frac{\Delta S_1}{R}}}. \tag{4}$$

Equations 3 and 4 show that the over-all intensity of luminescence, under optimal physiological conditions of pH, oxygen, salt concentration, etc., will be determined by the influence of temperature on two reactions involving the same molecule, namely, the rate of the catalytic reaction and the inactivation equilibrium of the enzyme, respectively.

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The values of the constants in EQUATION 4 may be estimated from experiments in which I is measured at different temperatures and pressures. Thus, the apparent $\Delta H^{\ddagger}$, which at normal pressure is indistinguishable from "μ" of the Arrhenius equation, is computed from the slope of the line relating $ln\ I$ and $\frac{1}{T}$ in the low temperature range where the value of K_1 is negligible. ΔH_1 is obtained from the slope of the decreasing I with increasing T at temperatures above the optimum, and adding to this the value of $\Delta H^{\ddagger}$, disregarding signs. $\Delta V^{\ddagger}$ may be determined from the slope of the line relating $ln\ I$ to pressure (p) in atmospheres at low temperatures. ΔV_1 is obtained from a similar plot at high temperatures, ΔE from the relation, $\Delta H = \Delta E + p\Delta V$, and ΔS from the relation

$$K = e^{-\frac{\Delta H}{RT}} e^{\frac{\Delta S}{R}} = e^{-\frac{\Delta E}{RT}} e^{-p\frac{\Delta V}{RT}} e^{\frac{\Delta S}{R}}.$$

These constants then make it possible to calculate, using EQUATION 4, the intensity of luminescence with some accuracy at various temperatures and pressures, as shown (FIGURE 1) by Eyring and Magee.[19] In this

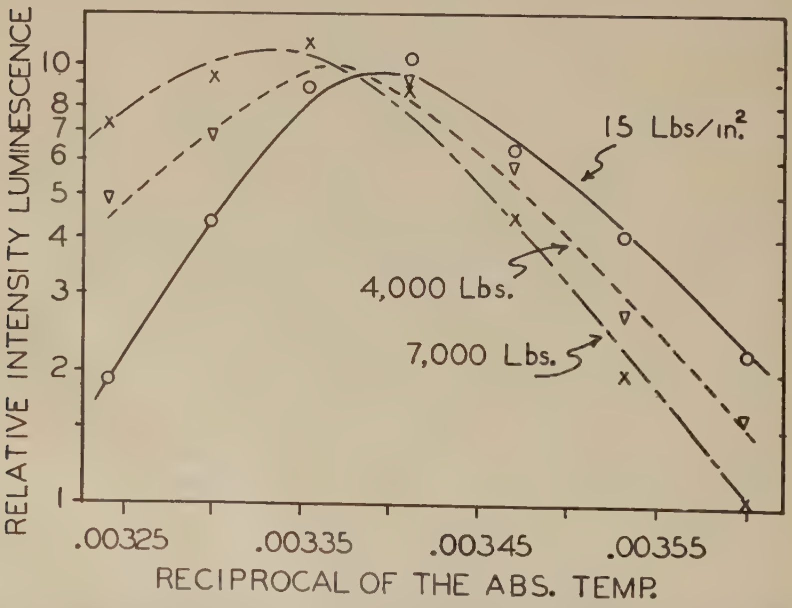

FIGURE 1. Relation between the intensity of luminescence (*Photobacterium phosphoreum*) and temperature, under normal and increased hydrostatic pressures of 4,000 and 7,000 lbs./sq. in., respectively. The solid lines are curves calculated by Eyring and Magee[19] as described in the text; the points represent data from experiments of Brown, Johnson, and Marsland.[17] Logarithmic scale on the ordinate.

case, $\Delta V^\ddagger$ amounts to 50 cc. per mole at 0° C, and ΔV_1 to 64 cc. at 35° C, but a temperature dependence of these constants has been taken into account. The constants employed are as follows: $\Delta E^\ddagger = 17{,}220$ calories, $\Delta V^\ddagger = 546.4 - 1.813\text{T}$ cc., $\Delta E_1 = 55{,}260$ calories, $\Delta S_1 = 184$ Entropy units, $\Delta V_1 = -922.8 + 3.206\text{T}$ cc.

The difference in the values of the constants for the luciferin-luciferase reaction and for the inactivation equilibrium of luciferase, respectively, are largely responsible for the optimal temperature of luminescence at a given pressure, and likewise for an optimal pressure at a given temperature. The apparent activation energy of 17,220 calories at atmospheric pressure is similar to, or not very different from, those familiarly associated with enzyme reactions and more complex biological processes as a whole. The heat and entropy of the inactivation equilibrium, as pointed out above, are typical of protein denaturation. The volume changes of activation and of reaction, respectively, are of particular interest, since both are high and show that in each case the reaction involves a very large molecule. These volume changes indicate that fairly drastic alterations in the structure of the molecule take place both in the formation of the activated complex and in the reversible inactivation. They probably indicate considerable unfolding of the protein from a somewhat globular to a more fibrous form. Thus, in the process of catalysis, it would appear necessary for the enzyme to change its configuration to fit the substrate, although such a change would not be necessary if the active or combining groups of the enzyme are at the surface, which appears to be the situation with invertase.[21]

Turning now to the action of inhibitors which combine reversibly with the luminescent system, it is evident from EQUATION 2 that the equilibrium through which the system is inactivated may be established either independently of the denaturation equilibrium (Type I) or in relation to it (Type II). The former mechanism has the likeness of a combination of the inhibitor with a prosthetic group of the enzyme, or possibly the luciferin. In this case, kinetic data alone are not sufficient to distinguish between the enzyme and substrate as site of action.[18] Type II represents a combination of the inhibitor with bonds which are involved in the denaturation equilibrium. Although Types I and II cannot be distinguished experimentally on the basis of the relation between concentration of inhibitor and the amount of inhibition observed, other conditions remaining constant, they may, in general, be expected to behave in different ways as pressure and temperature are varied. Thus, in Type I, letting K_2 represent the equilibrium constant, independent of K_1, pressure would be expected to have only slight influence, while a rise in temperature should result in a decrease in per cent inhibition, as the enzyme-inhibitor complex is dissociated and K_2 becomes smaller. Since the inhibition at a given drug concentration is less at lower temperatures, an increase in apparent activation energy of the luminescent reaction occurs in the presence of the drug, and the temperature of maximum luminescence is slightly increased. On the other hand, in Type II, with equilibrium constant K_3 because of its dependence on K_1, which is characterized by a large volume change of reaction, a pronounced effect of pressure on the inhibition would be anticipated. Thus, at temperatures near the normal optimum, moderate inhibitions by alcohol can be completely reversed by hydrostatic pressure,[6] as illustrated in FIGURE 2. The influence of pressure on the inhibition caused by various drugs, including representatives of both types, is illustrated in FIGURE 3.[22] As for temperature, the influence in this type depends on both K_1 and K_3, that is: although the enzyme-inhibitor complex dissociates with rise in temperature, the bonds with which the inhibitor combines may be more available; K_1 increases and K_3 decreases as the temperature is raised, and the net result depends on K_1 times K_3. In the cases studied, the per cent inhibition increases with rise in temperature, resulting in a lowering of the apparent activation energy, a decrease in the observed heat of denaturation, and a

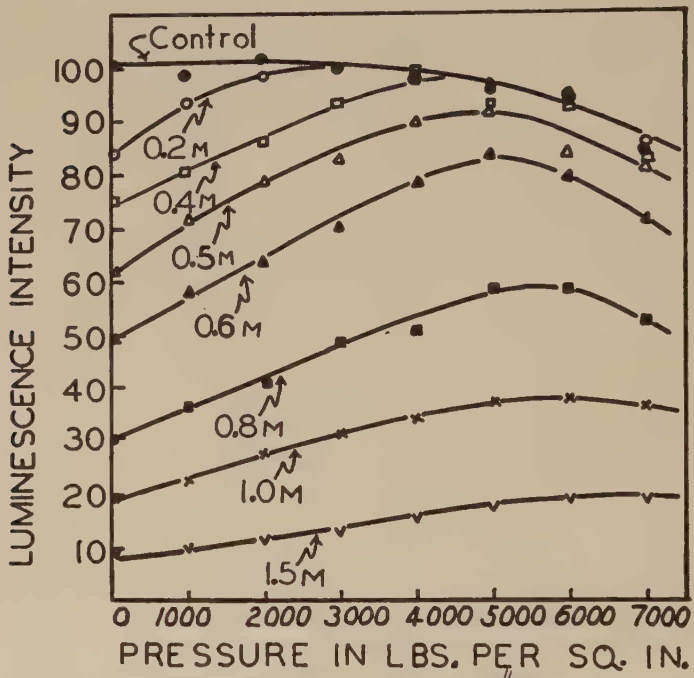

FIGURE 2. The relation between hydrostatic pressure and the amount of inhibition of luminescence at 17.5° C caused by the various concentrations of alcohol indicated on the figure.[6] (*Photobacterium phosphoreum.*)

somewhat lower temperature of maximum luminescence, in the presence of a given drug concentration.

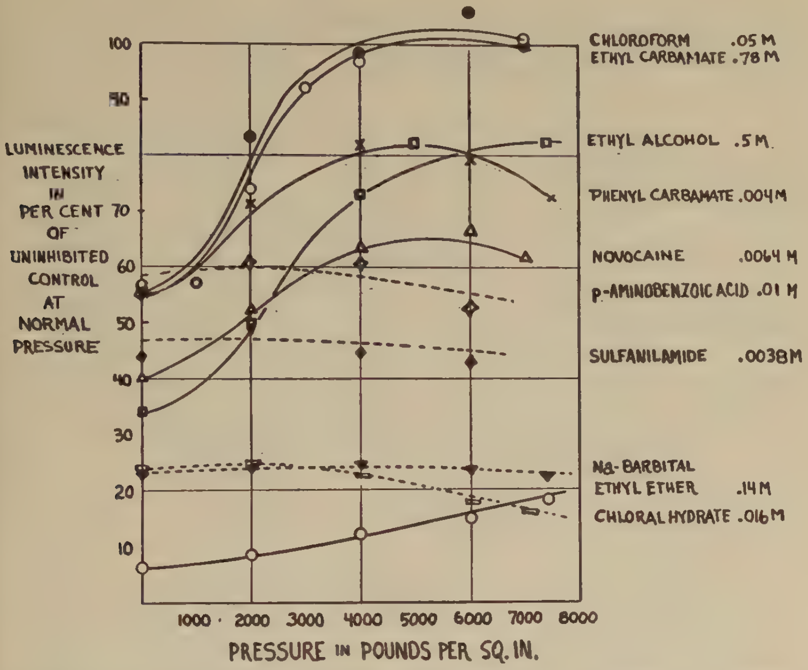

FIGURE 3. The relation between hydrostatic pressure and the amount of inhibition of luminescence caused by a given concentration of various drugs, at 17 to 18° C. The intensity of the drug-free control at atmospheric pressure has arbitrarily been taken as 100, and the other points computed in relation to it. (*Photobacterium phosphoreum.*[22])

The relation between luminescence intensity and temperature, without the addition of drugs, and in the presence of a single concentration of sulfanilamide and urethane, respectively, is illustrated in FIGURE 4.[6]

These respective mechanisms may be formulated, and expressions obtained for arriving at the constants, K_2 and K_3, from the data of experiments. Thus, letting I_2 represent the observed luminescence intensity in the presence of a given concentration of inhibitor, we have, for Type I,

$$I_2 = \frac{bk(LH_2)(A_0)}{1 + K_1 + K_2X^r + K_1K_2X^r}, \tag{5}$$

in which X represents the molar concentration of inhibitor, and r the ratio between the inhibitor and enzyme molecules in the complex formed. Dividing EQUATION 2 by EQUATION 5 and simplifying,

$$\left(\frac{I_1}{I_2} - 1\right) = K_2\, X^r. \tag{6}$$

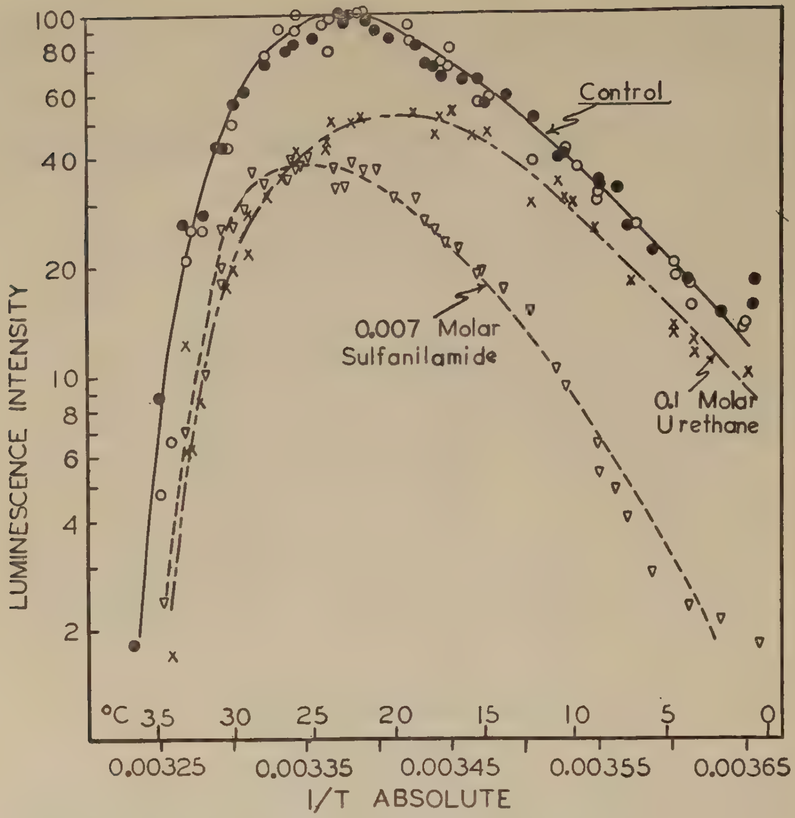

FIGURE 4. Luminescence intensity of *Photobacterium phosphoreum* in relation to temperature in a control and in corresponding suspensions containing 0.007 molar sulfanilamide and 0.1 molar urethane, respectively. The solid and hollow circles for the control are from repeated experiments. The data are replotted from **Johnson, Eyring, Steblay, et al., 1945.**[6]

Similarly, for Type II,

$$I_2 = \frac{bk(LH_2)(A_o)}{1 + K_1 + K_1K_3U^s}, \qquad (7)$$

in which U represents the molar concentration of inhibitor, and s the ratio of drug-enzyme molecules in the complex. Dividing EQUATION 2 by EQUATION 7 and simplifying,

$$\left(\frac{I_1}{I_2} - 1\right) = \frac{K_1K_3U^s}{1 + K_1}, \text{ or} \left[\left(\frac{I_1}{I_2} - 1\right) \left(1 + \frac{1}{K_1}\right)\right] = K_3U^s. \qquad (8)$$

With the aid of EQUATIONS 6 and 8, the two mechanisms may be distinguished. In either case, a plot of $ln \left(\frac{I_1}{I_2} - 1\right)$ against ln molar concentration of inhibitor at constant temperature and pressure, yields straight lines whose slope gives the average number of inhibitor molecules combining with each enzyme molecule (FIGURE 5), unless, of course, more than one system is affected or additional reactions are involved, so that the action is more complicated than the theory takes into account. When, on the other hand, $ln\ \left(\frac{I_1}{I_2} = 1\right)$ is plotted against the reciprocal of the absolute temperature, a straight line over a wide range of temperatures is obtained for Type I, but not for Type II (FIGURE 6). From the slope of the straight line, the heat of reaction, ΔH_2, of the equilibrium may be computed. If the mechanism is Type II, a straight line,

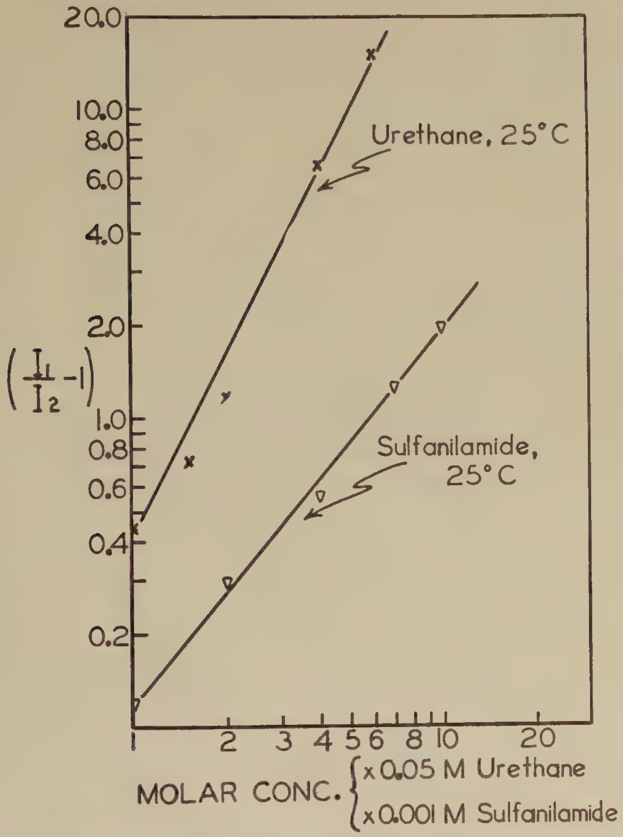

FIGURE 5. Relation between the concentration of sulfanilamide and urethane, respectively, and the amount of inhibition of bacterial luminescence, (*Photobacterium phosphoreum*) at 25° C., plotted in the manner discussed in the text.[6] The slope of the line for sulfanilamide is 1.2; for urethane, 2.0. With urethane, the slope increases with temperature, although with sulfanilamide there is little change.

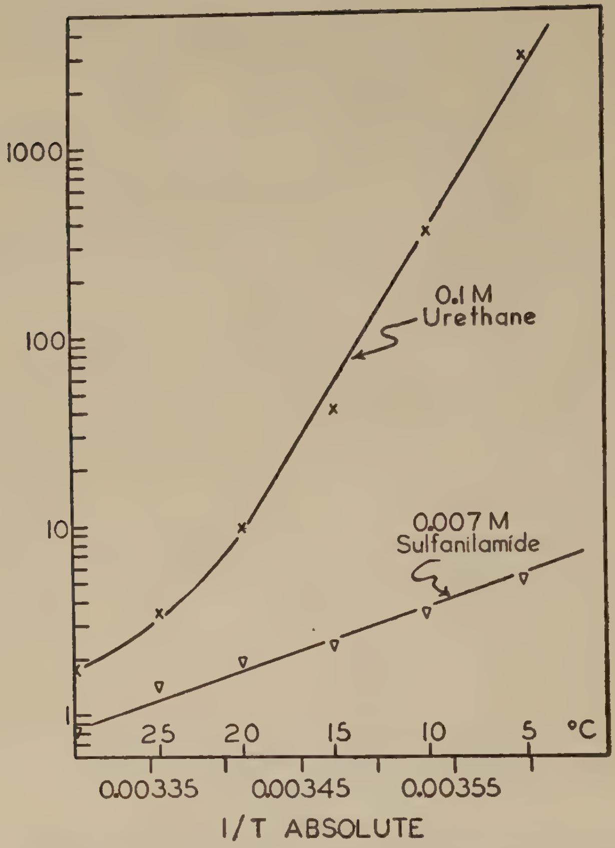

FIGURE 6. Analysis of the curves shown in FIGURE 4, according to the formulations given in the text. The luminescence intensity of the sulfanilamide-containing suspension (I_2) in relation to that of the control (I_1) is plotted on the logarithmic scale of the ordinate as $\left(\frac{I_1}{I_2}-1\right)$ against the reciprocal of the absolute temperature on the abscissa. The slope indicates 12,500 calories as the heat of reaction in the sulfanilamide-enzyme equilibrium. The values of the expression $\left(\frac{I_1}{I_2}-1\right)$ for the urethane curve in FIGURE 4 in relation to the control have been multiplied by $\left(1+\frac{1}{K_1}\right)$, and the product plotted on the ordinate for different values of $1/T$. In this case, the slope of the line, through the lower temperature range, indicates a heat of reaction of approximately 60,000 calories in the urethane-enzyme equilibrium.

whose slope depends on the heat of reaction, ΔH_3, results when $ln\left[\left(\frac{I_1}{I_2}-1\right)\left(1+\frac{1}{K_1}\right)\right]$ is plotted against $\frac{1}{T}$ (FIGURE 6). In the cases studied, however, the relation frequently departs from linearity, in the direction of too high an inhibition at temperatures beyond the normal optimum. Inasmuch as such deviations have been found to be more marked with increasing concentration of the drug, and since Type II inhibitors have been found to promote an irreversible as well as a reversible denaturation of the luminescent system,[6] it appears likely that, under these conditions, the irreversible effects are added to the reversible ones. Only the latter are taken into account by the above formulation.

In a purely diagrammatic way, the influence of temperature, pressure, and inhibitors of the two types discussed above may be illustrated as shown in FIGURE 7. Thus, at given concentrations of substrate and enzyme, the rate of the reaction in a single system, or in a complex process consistently limited largely by a single system, will be accelerated by a rise in temperature, and retarded by an increase in pressure, in a manner quantitatively determined by the specific reaction rate constant, k_1. The extent to which the rate increases for a given rise in temperature depends on the heat of activation, $\Delta H_1^\ddagger$, in the equilibrium, $K_1^\ddagger$, between the reactants and the activated complex, which, once formed, decomposes with the universal frequency, $\frac{kT}{h}$, characteristic of all chemical reactions. Likewise, the amount of the pressure effect depends on the volume change of activation, $\Delta V_1^\ddagger$, in the same equilibrium constant, $K_1^\ddagger$. As long as the enzyme molecules remain within the active channel, enclosed

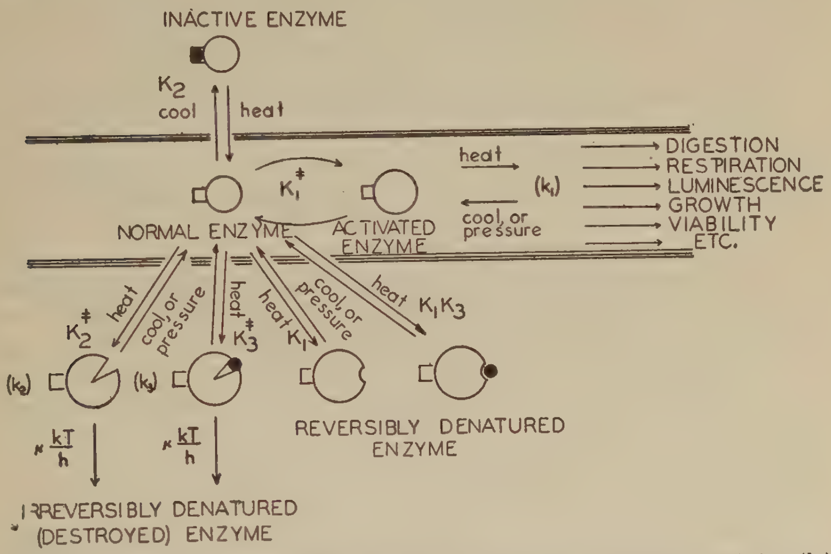

FIGURE 7. Schematic diagram to illustrate the control of the luminescent oxidation (k_1) in relation to temperature, hydrostatic pressure, reversible Type I inhibitors (K_2), reversible Type II inhibitors (K_3), the reversible temperature inactivation of the enzyme (K_1), irreversible thermal destruction (k_2), and irreversible destruction promoted by Type II inhibitors (k_3), as discussed in the text.

by the parallel lines (FIGURE 7), while the substrate concentration also is maintained at a constant, steady-state level and other factors remain the same, the reaction rate depends only on the value of k_1.

The amount of active enzyme, however, may be altered by one or more reactions. In the first place, as indicated above, it is apparent that the enzyme normally exists in equilibrium (K_1) between active and inactive forms. Because of the high heat and entropy of this reaction, the value of K_1 at temperatures below the optimum is so small as to be negligible. In the region of the optimum and above, K_1 increases rapidly with rise in temperature, causing the proportion of inactive molecules to increase to a greater extent, by a given increment in temperature, than the activation process in the enzyme reaction is accelerated: *i.e.,* K_1 is more strongly influenced by temperature than k_1. As a result, the observed rate goes through a maximum. Similarly with pressure: the inactivation proceeds with a large volume increase and, as a consequence, K_1 is decreased with rise in pressure and is more markedly affected than k_1, other factors remaining the same.

In addition to the reversible inactivation, with an equilibrium constant indicative of a protein denaturation equilibrium, a rate process of thermal destruction also takes place. In luminescence, the latter reaction has an even higher activation heat and entropy than the ΔH and ΔS of the reversible denaturation (FIGURE 8).[6] It is for this reason that the reversible denaturation of the luminescent enzyme can be so readily observed experimentally. The thermal destruction also proceeds with a large volume increase of activation, and consequently is markedly retarded by pressure at a given temperature (FIGURE 9).[6]

Both the reversible and irreversible denaturations are furthered by the addition of inhibitors of Type II, such as urethane or alcohol, apparently by the combination of these agents with bonds made available in the denaturation reaction. Both are opposed by hydrostatic pressure or by cooling.

By a mechanism different from those just described, certain inhibitors (Type I) enter into an equilibrium, K_2, causing an inactivation of the enzyme independently of the denaturation. The combination apparently occurs equally with the active and reversibly denatured forms and does not itself lead to an alteration in structure of the protein accompanied by a large volume change. Consequently, pressure has little influence. On the other hand, the enzyme-inhibitor complex will, in general, be dissociated by a rise in temperature, so that the fraction of inactive molecules will be less at higher than at lower temperatures, and the per cent inhibition correspondingly smaller.

The generality of the mechanisms, as shown in FIGURE 7, to a large extent awaits justification by further experiments, inasmuch as data sufficient for analysis on the basis of the theory described are not widespread,

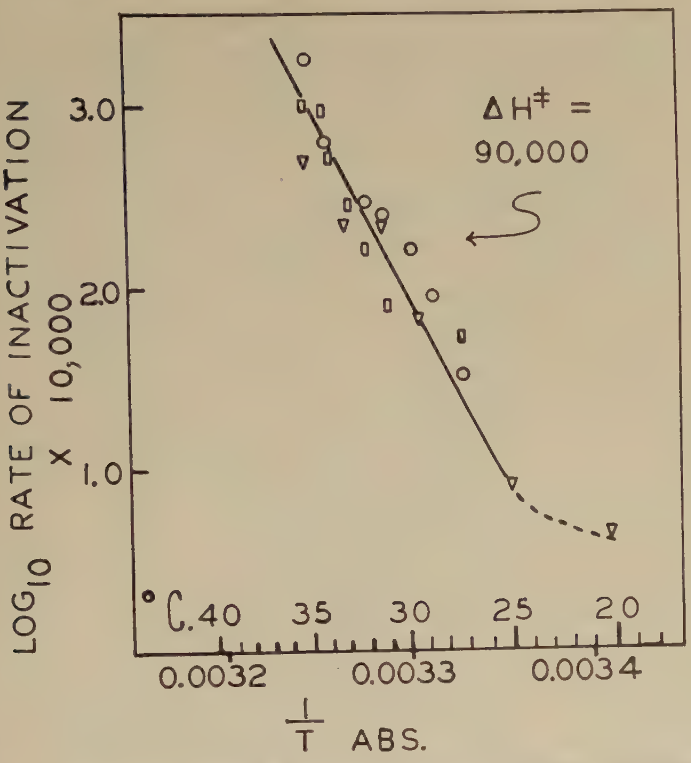

Figure 8. Temperature analysis of the rate of thermal destruction of the luminescent system in bacteria (*Photobacterium phosphoreum*). The points are from three repeated experiments. The slope of the line indicates an apparent activation energy of some 90,000 calories.[6] Semi-log scale.

particularly with respect to the action of hydrostatic pressure. Moreover, previous to the studies in connection with luminescence, the possibility that protein denaturation might be opposed by hydrostatic pressure had apparently not been taken into account. The opposite effect has been known for some years, *viz.*, that very high pressures, of the order of 10,000 atmospheres, at room temperature denature proteins, kill microorganisms, and inactivate enzymes, viruses, antibodies, bateriophage, etc. (*cf.* reviews by Macheboeuf and Basset[23] (1934), Cattell[24] (1936), and Bridgman[25] (1946). It is perhaps worth while, therefore, briefly to consider some of the available evidence with respect to other systems than luminescence.

First of all, it is apparent that the reactions by which proteins are denatured at room temperature under very high pressures are not identical with the reactions which take place at relatively high temperatures (or at lower temperatures in the presence of certain drugs such as alcohol) and which are opposed by moderate pressures, of the order of 500 atmospheres.

The fact that the lower pressures may greatly retard the denaturation of a highly purified protein, human serum globulin, at 65° C. has recently been demonstrated.[26] Small concentrations of ethyl alcohol accelerate the precipitation, while pressure retards it, with alcohol as well as without it (FIGURE 10). The rate is higher than first order. Preliminary estimates of the volume change indicate a volume increase of activation of about

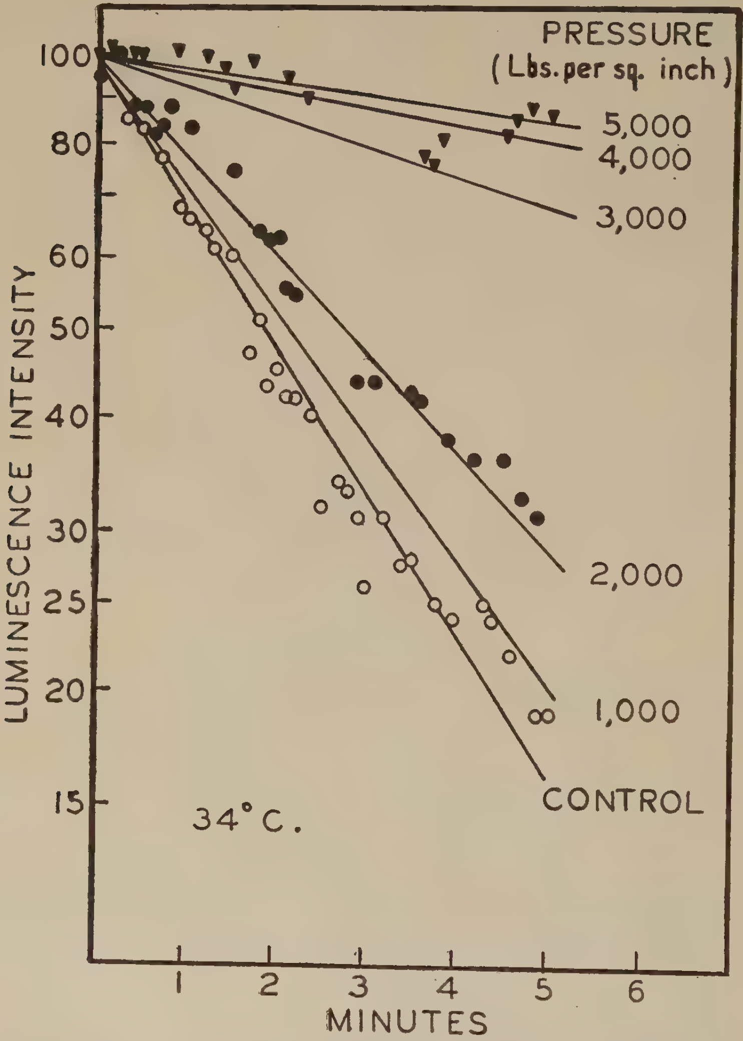

FIGURE 9. Influence of hydrostatic pressure in retarding the thermal destruction of the luminescent system at 32° C.[6]

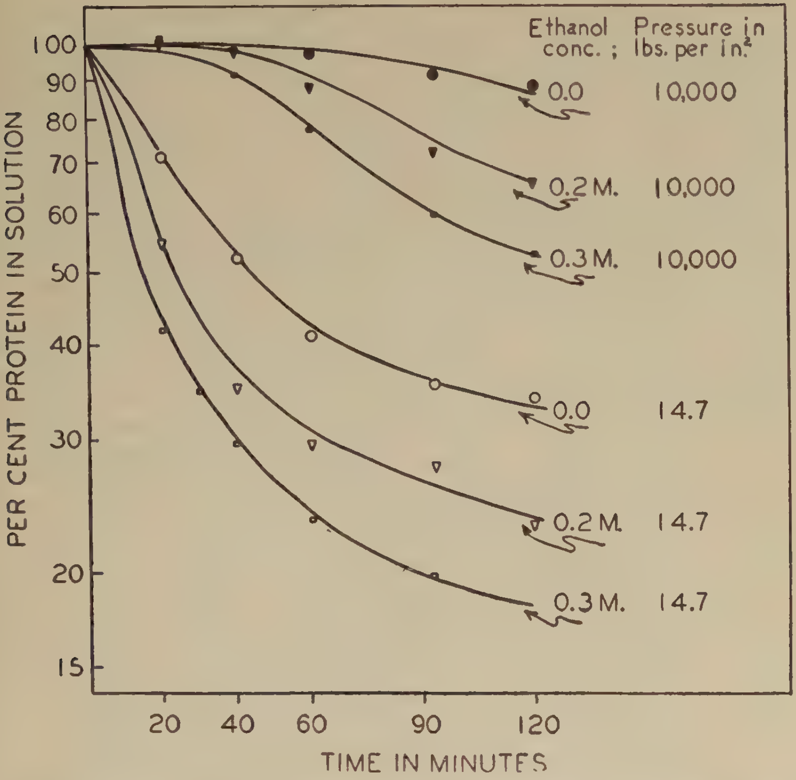

FIGURE 10. The rate of precipitation of highly purified human serum globulin at 65° C. in relation to hydrostatic pressure and small concentrations of ethyl alcohol.[26] Semi-log scale.

100 cc. per mole. The denaturation of specific antitoxic activity at the same temperature is also opposed by a pressure of 680 atmospheres.[27] Furthermore, specific precipitation of rabbit immune serum by a simple trihaptenic dye antigen, at room temperature, is greatly retarded under pressure of 680 atmospheres, indicating a large volume increase, of some 50 cc. per mole according to available data, in the reaction involving the antibody molecules.[25]

In regard to extracted enzyme and sol-gel systems, Marsland and Brown[29] have shown that the sol-gel equilibrium of rabbit myosin is characterized by a volume change of 120 cc. per mole. This is especially interesting, since sol-gel reactions influencing intracellular processes involving protoplasmic streaming, *e.g.* cyclosis, amoeboid motion, cleavage of animal cells, etc.,[30] are also accompanied by large molecular volume changes, of the order of 102 cc. per mole.[29] The rate of various extracted enzyme systems, *e.g.*, lipase, pepsin, and pancreatic proteinases (Benthaus, 1942[31]; *cf.* also Deuticke and Harren, 1938[32]), at room temperature, is reversibly retarded by pressures up to 1,500 atmospheres. As yet, sufficient data do not exist for a satisfactory analysis of the volume changes in these enzyme reactions. Thus, further studies should yield interesting results. Moreover, it might be expected that, under conditions favoring a reversible denaturation of the enzyme, an increase in the net activity of the system under pressure would be encountered, as in luminescence.

The activity of invertase or of diastatic enzymes is apparently not greatly retarded under moderate pressure at room temperature (Regnard, 1884[33]) and may even be increased.[21, 31] Recent studies[21] have shown that the increase in rate of sucrose inversion by invertase is most marked under conditions of partial inactivation of the enzyme, such as high temperatures at optimum pH, or at lower temperatures in relatively alkaline or acid solution (FIGURE 11). Analysis of the data indicated that at pH 7.05 and 35 or 40° C., the enzyme undergoes a reversible denaturation with a volume increase of about 69 cc. per mole.

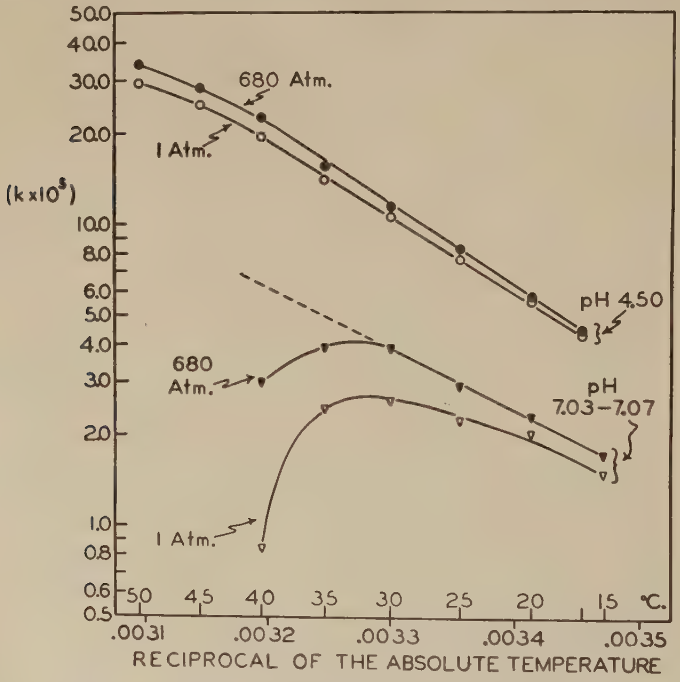

FIGURE 11. The rate, during the logarithmic period, of inversion of 10 per cent sucrose by invertase, in relation to temperature and hydrostatic pressure, at pH 4.5 and pH 7.03-7.07, respectively.[21]

In living cells, a number of phenomena, such as the tension of auricle muscle at room temperature, increase in intensity under pressures up to about 400 atmospheres, and then decrease as the pressure is raised up to less than 1,000 atmospheres.[34] The similarity in effects of pressure on these processes and on luminescence suggests that corresponding mechanisms are involved. In still other complex phenomena, including the rates of microbial growth and disinfection, a reversible inhibition by pressure has been noted.[35] A greater multiplicity of reactions are, no doubt, concerned in bringing about the measured result in all these cases, and the analysis, therefore, is more difficult than in luminescence.

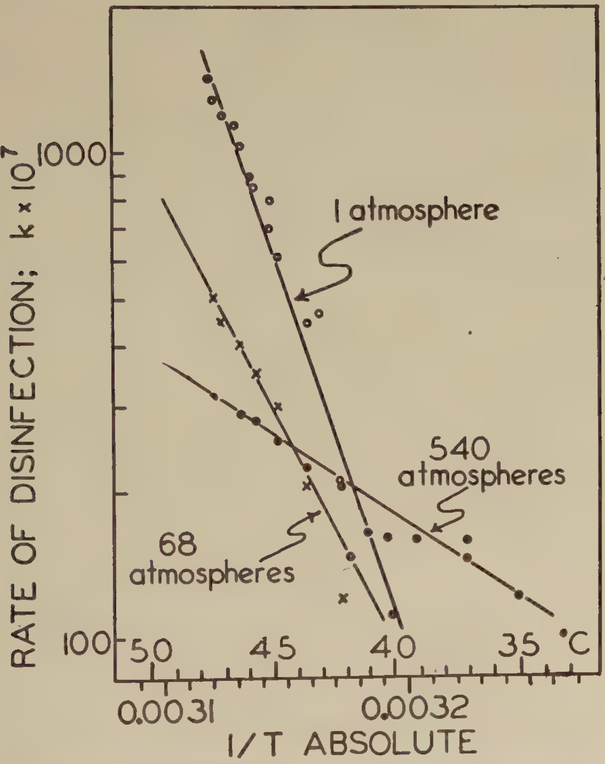

FIGURE 12. The rate of disinfection of non-proliferating cells of *Escherichia coli* in relation to temperature, under normal and increased hydrostatic pressures of 1,000 and 5,000 pounds per square inch, respectively.[35]

FIGURES 12 and 13 illustrate some of the data pertaining to growth and disinfection.

The relation between hydrostatic pressure and amount of inhibition caused by a given concentration of a drug has apparently been studied only in connection with bacterial luminescence,[6, 22, 36] and to a lesser extent, bacterial growth and disinfection.[35] Although the influence of

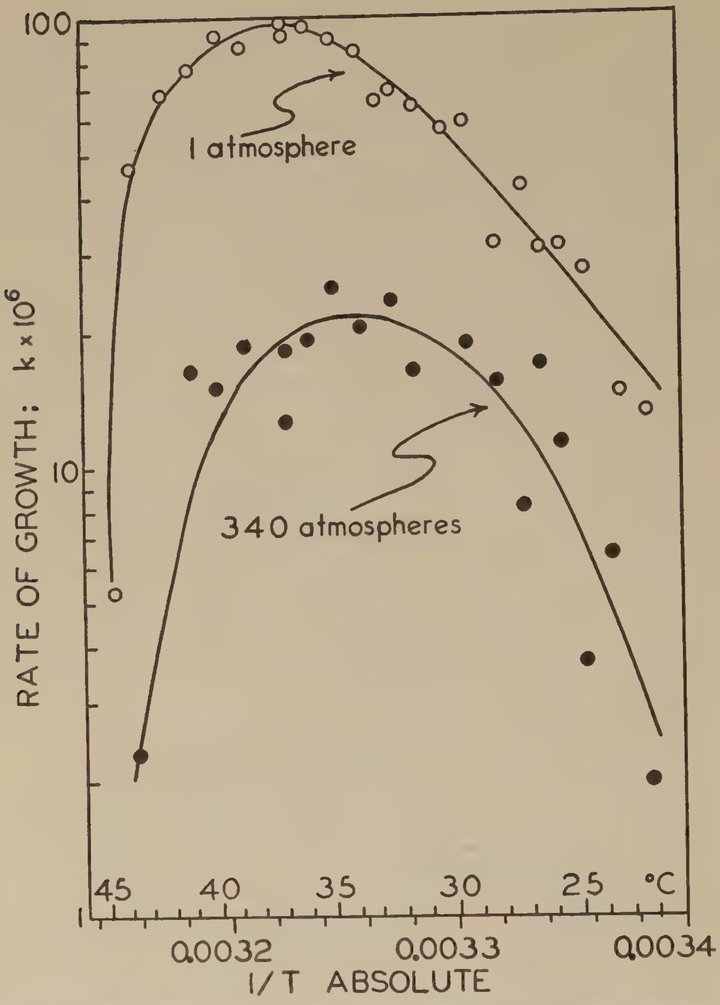

FIGURE 13. Growth (reproductive) rate of *Escherichia coli*, during the logarithmic phase, in relation to temperature, under normal and increased hydrostatic pressure of 5,000 pounds per square inch.[35]

temperature, at normal pressure, has received considerable attention, the data are not often sufficiently extensive, nor the mechanism of the reactions sufficiently well understood, to justify undertaking an analysis on the basis of the theory described above. According to the results of a recent investigation,[37] however, it appears that the rates of oxygen consumption and of methylene blue reduction by *Rhizobium trifolii* are affected by urethane in a manner resembling the action of this drug on bacterial luminescence. Using the same formulations, the quantitative relation between various concentrations of the drug, and the amount of inhibition at various temperatures, may be accounted for with some accuracy. The influence of pressure in this case has not been studied, but an analysis of its action should assist in elucidating the mechanism of the inhibition.

The evidence that large volume changes of reaction or of activation, respectively, take place in such diverse phenomena as luminescence, extracted enzyme reactions, cell division, protein denaturation, specific precipitation, the action of certain inhibitors, and so forth, would seem to justify more extensive studies from the point of view of hydrostatic pressure as well as temperature. Furthermore, there is considerable reason to believe that the synthesis of complex molecules, biologically specific in structure, involves a templet mechanism, and in order for a molecule to act as a templet it must be in a one- or at most two-dimensional form. This means that globular molecules would have to unfold before functioning as a templet. Such unfolding might be expected to be accompanied by fairly large volume changes. Studies with hydrostatic pressure should yield significant data in this connection also, and the theory worked out with luminescence as an indicator of protein reactivity will perhaps be found useful in various connections.

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THE ANATOMY AND PHYSIOLOGY OF THE LIGHT ORGAN IN FIREFLIES*

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* The histological and cytological work which forms the basis for the original photomicrographs, and for various critical opinions appearing in this paper, was begun in 1938 at the Department of Embryology, Carnegie Institution of Washington, Baltimore, Md., and was continued at the University of Rochester during the period of 1939-1945. The author acknowledges with pleasure the technical assistance of Mrs. Gustav Kuerti, Miss Margaret Ramsay, Miss Olive Pitkin, and Miss Margaret Keister in the preparation of some two thousand microscope slide preparations, part of which was made possible by a grant-in-aid from the Society of the Sigma Xi. Grateful acknowledgment is likewise due to the National Research Council, and to the American Philosophical Society for grants which made possible the author's participation in the 1936 and 1941 expeditions to Jamaica under the auspices of The Johns Hopkins University. Thanks are due also to Miss Inez Demonet and associates for preparing the drawings for this report, and particularly to Dr. Rubert Anderson, Dr. Leigh Chadwick, Dr. E. N. Harvey, Dr. H. Specht, and Dr. Carroll Williams for extensive and helpful suggestions. Appreciation is due to the University of Rochester for generous help with the costs. Finally the author is enormously indebted to Elizabeth Mast Buck for aid with the manuscript.

INTRODUCTION

Since the days of Aristotle and Pliny, and presumably long before recorded scientific observation, the mystery of organic light has aroused the wonder of mankind. Fireflies being by far the most easily accessible luminous organisms, it is understandable that they were probably the earliest and most frequently studied forms. They are also outstanding in their own right, as a subject for scientific observation and experimentation, for perhaps no other animals have luminous organs of such size, brilliance, intricate structure, and physiological complexity. Therefore, it is not surprising that in the past two or three centuries an enormous literature has grown up on various aspects of firefly and glowworm luminescence. It is interesting to see that the problem has proved irresistible not only to specialists on bioluminescence but to a surprising number of men illustrious in other fields, as, for example, Swammerdam, Spallanzani, Davy, Faraday, Humboldt, Darwin, and Pasteur. In this paper, I shall not attempt to cover the literature exhaustively, but rather to review critically the major contributions bearing on the circumscribed problem outlined below.

As our knowledge of living light has increased, and the type of research has shifted from the naturalistic to the quantitative, it has turned out, for one reason or another, that certain aspects of the problem are better investigated on luminous organisms other than the firefly. Thus the crustacean *Cypridina* and luminous bacteria, in particular, have been used in most modern work on the chemistry, kinetics, and enzymology of bioluminescence. For the study of the isolated systems, the very complexity of the firefly light organ is, in a sense, a hindrance. On the other hand, it is well to recall that much of the pioneer work on bioluminescence, even on *in vitro* systems, was done on fireflies. Spallanzani (1796) used the glowworm in one of the earliest demonstrations that luminescence is dependent upon water and oxygen. In the tropical beetle *Pyrophorus*, Dubois (1886) first distinguished the enzyme and substrate of animal luminescence. It was, finally, on fireflies that Coblentz (1912) completed what still stands as the most comprehensive work on the spectroscopy and photometry of light-emission in living organisms. Moreover, fireflies still form the preeminent material for the study of at least two aspects of bioluminescence: the intimate anatomy of photogenic organs, and the physiology of the control of luminescence. This presentation will concentrate on these two aspects.

Since the main task of this paper is the integration of the structural details of the firefly light organ with the various manifestations of light production, it may be helpful briefly to outline the problem from the theoretical side, so that we may have a clear idea of what sort of information will be needed. The most fundamental level of the problem is the chemistry of the luminescent reaction. We need not be concerned with this, except to know the reactants. The next level of the problem concerns the general anatomy of the region in which the luminescent reaction takes place. Ideally, this would involve knowing where the different reactants are formed or obtained, how they are brought to the scene, and where they are held pending the reaction, or stored if in excess. The final level is the occurrence and control of luminescence. For this, we will need to know how the reactants are brought together at the desired moment, exactly where the reaction occurs, what sorts of luminescences are possible, which of the reactants is made limiting in stopping the reaction, and what physiological mechanisms operate in this control. For the present, we must be content with very incomplete knowledge at each level.

A long series of researches, initiated by Dubois (1885-1919)* and given quantitative expression by Harvey and his associates (Harvey, 1920 and 1940), show that the photogenic reaction involves a minimum of four reactants: water, oxygen, the substrate luciferin (a substance of low molecular weight, probably phenolic), and the enzyme luciferase. For most purposes, it makes little difference whether the oxygen is actually used in the energy-liberating reaction or reactions or in the recovery process.

THE ANATOMY OF THE LIGHT ORGAN

Gross Anatomy. Even externally, the luminous organs of the firefly are so diverse that the only generalizations possible are that they are close to the body surface behind a window of translucent cuticle, and are usually different in male, female, and larva. In size, the organs vary from minute pin-heads to masses occupying the entire ventral surfaces of several abdominal segments. In outline they vary from circular to entirely irregular. In position, they are found from the head to the tip of the abdomen, including the thorax, and on both dorsal and ventral surfaces. A number of representative luminous beetles are shown in FIGURE 1. Most of our common fireflies *(Photinus* and *Photuris)* are of the sort in which the organs occupy sternites 6 and 7 in the male (FIGURE 1, *a*), variable portions of 6, or occasionally 6 and 7, in the female (FIGURE 1, *b* and *e*), and two small spots on the ventral surface of sternite 8 in the larva (FIGURE 1, *c*). This larval position is the same as that in the adults of both sexes of the tropical genus *Diphotus* (FIGURE 1, *d:* Barber, 1941), which fact raises evolutionary questions. Another interesting type is that illustrated by the common European glowworms *Lampyris noctiluca* (FIGURE 1, *r*) and *Lamprorhiza (Lampyris) splendidula*† (FIGURE 1, *o*),

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* In this paper, reference is made only to **Dubois'** 1886 monograph. Citations of his many other papers can be found in **Mangold** (1910) and **Harvey** (1920).

† The taxonomy of fireflies is in a very confused state. Among the points bearing on the subject matter of this paper are the following: (1) Many fireflies, to which the generic name *Lampyris* was applied originally, have since been put in other genera and hence are cited under different names in the older and in the more recent literature (examples: *Lamprorhiza splendidula; Luciola italica,* sometimes put in *Phausis; Luciola lusitanica; Phosphaenus hemipterus.* (2) There exist both *Photinus marginellus* and *Photinus marginellatus.* (3) The famous tropical firefly *Pyrophorus* is an elaterid (click-beetle) and is in a different family from all the other "fireflies" (the beetle family Lampyridae). (4) Because of inadequate descriptions in the literature, **Barber** was unable to identify, in my collections, most of the lampyrid species previously reported from Jamaica. It is probable, therefore, that some of the species used by **Lund** (1911) were actually studied by me under new names given by **Barber** (1941). I have followed custom in using the spellings "*pennsylvanica*" and "*Lamprorhiza*" for the taxonomically correct "*pensylvanica*" and "*Lamprohiza.*"

on which much work has been done. In these species, the most striking luminosity is seen in the wingless female. Another and very different type of organ distribution is seen in the famous tropical elaterid *Pyrophorus* (FIGURE 1, *m,n*). Both sexes of this beetle have small circular organs on the dorsal posterior corners of the pronotum, which emit a green light when the insect is at rest or walking, and a large rectangular organ in a cleft on the anterior surface of the abdomen, which emits an orange light when the insect is in flight. Some of the most spectacular displays of luminescence are seen in the larvae and larviform females of species in which the adult male is almost or entirely non-luminous. Such a creature is *Phengodes* (FIGURE 1, *s*), which has 11 or 12 segmentally arranged sets of photogenic organs giving a bright green light, and also its South American relative *Phrixothrix* (Harvey, 1944 and 1945), which has, in addition, a red light in its head.

Histological Types of Light Organ Structure. Firefly light organs show an astonishing diversity of structure and can be classified in a number of arbitrary ways. The system used here is an extension of Dahlgren's (1917), based on the arrangement of the tracheae.*

Typically, the photogenic cells are grouped together in one or more compact localized masses with specific tracheal and nervous supplies. However, one exception is found in *Phengodes,* (Type 1), where the light is produced by loose independent giant cells, apparently without tracheae, and similar to or identical with the oenocytes which are widely distributed among and within insects (FIGURE 15; see also Buck, 1940, 1942, 1946a).

The next more complex type of light organ structure is Type 2, found, for example, in *Phrixothrix* (Buck, 1946a), in the lateral "tuberculate" organs of the female of *Lamprorhiza splendidula* (Wielowiejski, 1882; Bongardt, 1903), and the larva of *Phausis Delarouzeei* (Bugnion, 1929). Here (FIGURE 16) the organs are small subspherical compact masses of polyhedral photogenic cells with typically granular cytoplasm, constant in location, and with a specific tracheal supply. This latter consists simply of progressively tapering and more numerous branches, which form a sort of root-system ramifying through the photogenic tissue (FIGURE 8).

The third type of organ is like Type 2, except for the presence of a second layer of cells on the inner surface of the photogenic tissue. This layer is the so-called "reflector layer". Since it will be discussed later, it will suffice at the moment to state that it is composed of cells differing sharply from the photogenic cells in appearance, chemical composition, and staining reaction. This type of organ is illustrated for the larva of *Photuris pennsylvanica* (FIGURE 17) and for an adult *Diphotus montanus* (FIGURE 19) and is also characteristic of the larvae of *Luciola cruciata* and *Pyrocoelia rufa* (Okada, 1935a and b; Hasama, 1942c), *P. analis*

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* The profuseness of the tracheal supply external to the photogenic organ has been mentioned many times and is well shown by Geipel (1915) and Hess (1921).

(Hasama, 1942c), *P. fumosa* (Hasama, 1944b), the larval organs of *Lampyris noctiluca* (see Wielowiejski and Bongardt), *Lamprorhiza splendidula* and *Phosphaenus hemipterus* (see Bongardt). The often-studied organs of the adult (female) of *Lampyris noctiluca* are also apparently of Type 3 (Owsjannikow, 1868; Wielowiejski; Bongardt). In addition, it seems clear, from the work of Heinemann (1886), Dubois (1886 etc.), Geipel (1915), Dahlgren, and others, that both the thoracic and abdominal organs of *Pyrophorus* show the compact but unorganized photogenic and reflector layers characteristic of Type 3, although the much larger size of the organs and much greater thickness of the layers tends to obscure the relationship (FIGURE 18). *Pyrophorus* also agrees with the abovementioned forms in having the simple arborescent tracheal supply seen in Type 2, in which the ultimate tracheal capillaries or tracheoles terminate between the photogenic cells and form the last link in an uninterrupted conduction system leading from the outside (FIGURE 8). The vertical spaces in the photogenic layer shown in Dubois' figure (FIGURE 12) have been doubted by Lund (1911) and Williams (1916) and are probably an exaggerated portrayal of the tendency, noted also by Dahlgren, of the photogenic cells to align in columns. The spaces could be caused artificially by these columns shrinking away from the tracheae running between them (which Dubois failed to see).

All the preceding types of organs differ from those now to be described in that they lack "tracheal end-cells." These cells, which were first described in fireflies by Schultze (1865), are structures which occur at the points where small tracheal twigs narrow rather suddenly and then divide into two or more delicate thread-like tracheoles, or tracheal capillaries, which *appear* to lack the spirally thickened walls which are characteristic of the tracheae proper. These end-cells seem to differ considerably in different material, or according to various workers, as is seen in FIGURES 2, 3, and 11 from Bongardt, Geipel, and Dahlgren, respectively. Nevertheless, there seems to be general agreement that they are uninucleate and have protoplasmic processes or extensions which surround and accompany the tracheoles for varying distances toward or among the photogenic cells, and give the end-cell a stellate appearance, which has led to their being compared with ganglion cells (Kölliker, 1858; Schultze; Eimer, 1872). It also seems clear that the end-cell is the principal site of reduction of inspired osmium tetroxide ("osmic acid") vapor, and, indeed, most of our knowledge of the end-cell was obtained by the use of this reagent (FIGURES 2, 3, and 11). An analysis of the significance of this reduction, and additional details on the internal structure of end-cells, are best deferred. It is worth pointing out, however, that end-cells are widespread in insects, and are even said, by Wielowiejski and Geipel, to occur in non-photogenic tissues of fireflies.

Among organs containing tracheal end-cells, there are a number of different anatomical arrangements which can be classified into convenient, though arbitrary, types. The simplest of these (Type 4) is reported by Dahlgren to occur in "some forms of *Photuris*" (though it does not appear to occur in either *P. pennsylvanica* or *jamaicensis*). As shown in FIGURE 4, the tracheae run ventrally through the reflector layer, as usual. However, when they reach the photogenic tissue, instead of penetrating the latter they divide into several branches which run laterally at the interface between the two layers, terminate in end-cells, and then send tracheoles down into the photogenic tissue, which is only one cell thick.

Another type (Type 5, see FIGURE 5) is found, according to Dahlgren, in the Japanese *Luciola parva, L. vitticollis,* and other oriental forms. The tracheal supply arboresces among the photogenic cells as in Types 2 and 3, except that, at certain points where the repeated branching and tapering has reduced the tracheae to quite small tubes, end-cells occur and give off the usual tracheoles. Thus defined, it agrees fairly well with the descriptions of the adult organs of *Pyrocoelia rufa* (Hasama, 1942a) and *Luciola africana* (Geipel), and seems also to include the older descriptions of the adult organs of *Lamprorhiza splendidula* (Schultze, Wielowiejski, Bongardt). The organ of *Luciola cruciata* (Okada, 1935b) is also placed in Type 5 provisionally, although in this organ (FIGURE 9) the larger tracheae appear not to be in direct contact with the photogenic cells, and the arrangement of the end-cells and tracheoles in horizontal section is more regular than expected (FIGURE 9b).

The Type 6 arrangement (FIGURES 7 and 10) is very common, being found in all American members of *Photinus* and *Photuris* which have been studied (*Photinus marginellus*—Townsend, 1904; Dahlgren. *P. pyralis*—Seaman, 1891; McDermott and Crane, 1911. *P. consanguineus* —Williams; *Photuris pennsylvanica*—Seaman; Lund; McDermott and Crane; Williams; Hess, 1922); in some twenty species of Jamaican *Photinus* (Lund; Buck, 1940 and 1942); in *Luciola parvula* (Hasama, 1944b); *Luciola italica* (Tozzetti, 1870; Emery, 1884); and doubtless many others. It is desirable to describe this type in some detail, in order to establish an adequate morphological basis for the future discussions on physiology. As a rule, the Type 6 organs are large, and situated on the ventral surfaces of sternites 6 and 7 in the male and 6 in the female (FIGURE 1, *a,b*). The reflector layer of the organ is in the inner or dorsal position, while the photogenic layer is ventral (FIGURE 20).

In Type 6, the tracheal trunks which supply the light organ run vertically through the reflector layer as usual, but when they reach the photogenic layer they do not pass directly between the photogenic cells but into specialized cylindrical rods of tissue which go straight through the photogenic layer to its ventral surface (FIGURES 7, 10, 23-26, 31, and 32).

These "cylinders"* contain a number of structural elements in addition to their axial tracheal trunk. First, there are short tracheal "twigs" which branch from the vertical trunk. Second, there is the tracheal epithelium, which, though extremely thin, has fairly large nuclei both along the trunk and the twigs. Third, there are tracheal end-cells, one at the end of each twig, with a nucleus about the size of those of the tracheal epithelium. Fourth are the tracheoles or tracheal capillaries which issue from the end-cells at just about the periphery of the cylinder and run out into the photogenic tissue. Though the existence of a differentiated membrane surrounding the cylinder is not readily apparent in many forms, some preparations suggest such a structure (FIGURES 29 and 38). The integrity of the cylinder tissue as a morphological unit is shown by the behavior of isolated cylinders in maceration preparations from which the photogenic cells have been removed (FIGURES 27, 37, and 40). In addition to these structures, there presumably is some sort of fluid or gelatinous matrix filling the apparent spaces in the cylinder around the end-cells, twigs, etc. Strangely enough, this material does not stain with any technique yet devised, so that in sections of the photogenic layer it is easy to come to think of the clear space around the tracheal trunk as "empty", and of the cylinders as hollow tunnels penetrating the photogenic tissue. If these spaces were indeed gas-filled, our ideas of how the photogenic tissue is supplied with oxygen would need revision.

In horizontal section (*i.e.*, one parallel to the external surface of the photogenic layer), or in surface view, the Type 6 organ shows a beautiful and characteristic "rosette" pattern, which has been remarked by numerous workers (Emery, Townsend, Geipel, Okada, etc.; FIGURES 10, 13, 22, 29, and 36). The vertical tracheal trunks are spaced in regular triangular symmetry, and around each the photogenic cells are arranged like the petals of a flower. In most species, the photogenic cells front upon two contiguous cylinders and have a roughly prismatic or rhombohedral shape.

The Type 6 organ has a number of interesting variations. For example, in many species, the cylinder often flares out like a trumpet or an hourglass at one or both surfaces of the photogenic layer, and there accommodates especially rich brushes of tracheal twigs and end-cells (FIGURES 28, 30, and 39). Such a cylinder in *Photuris jamaicensis* appears in FIGURE 25 in longitudinal section and is well shown in ventral surface view by Okada (1935b). Lund described the photogenic layer of the female of *Photuris pennsylvanica* as a single layer of cells, through which the tracheae run directly without cylinders, and which is penetrated by tracheoles (from end-cells) only from the dorsal and ventral surfaces (FIGURE 14). Such a structure is often seen. In other regions, however, even when only one cell thick, conventional cylinders occur with end-cells from which tracheoles run laterally. Possibly, Dahlgren's photurid organ (Type 4) was based on a similar structure in which the end-cells at the ventral surface were overlooked.

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* Called "digitiform acini" by Tozzetti and by Emery.

The arrangement in *Photinus pallens* is, in one sense, the reverse of that just discussed, in that its cylinder is commonly narrow at dorsal and ventral surfaces but expanded in the interior of the photogenic layer (FIGURE 26). Other species present numerous variations in the relative thickness of photogenic and reflector layers, number of cell layers in each, diameter of cylinders, etc. A few of those occurring in various species of *Photinus* are illustrated in FIGURES 23, 24, and 31. One of the most unusual of these is the organ of *Photinus evanescens montego,* which has an extraordinarily broad and short cylinder, with profusely branched tracheae. A high-power view of this organ shows well the terminations of the numerous tracheal twigs and the limits of the cylinder tissue (FIGURE 31).

One rather puzzling feature of the Type 6 organ concerns the relationship between the processes of the end-cells and the cylinder walls. In *Luciola italica* (Emery), *Photinus consanguineus* (Townsend, Dahlgren), *Luciola parvula* (Hasama, 1944b), and in all of the American and Jamaican species of *Photinus* which I have examined, the end-cells are apparently as figured by Townsend and Dahlgren, and seem to be wholly contained within the cylinder and to give off tracheoles of uniform diameter which penetrate the photogenic tissue alone. In *Photinus marginellatus,* however, which has been studied carefully by Geipel (FIGURES 3 and 6), in *Photuris pennsylvanica,* according to Lund, and in both *P. pennsylvanica* and *P. jamaicensis,* according to my observations, the end-cells appear to have quite thick tapering processes which enclose at least the proximal parts of the tracheoles (FIGURE 32). These processes actually penetrate varying distances into the photogenic tissue, even though the end-cell body itself may lie within the confines of the cylinder or in little bays in the photogenic tissue (FIGURE 14). The arrangement, therefore, resembles in some respects that described in *Lamprorhiza* (Type 5) by the earlier workers (FIGURE 2), although there is no doubt that both *Photinus marginellatus* and *Photuris pennsylvanica* have the vertical cylinders characteristic of Type 6. The matter will be considered further under the discussion of end-cells.

The "Reflector" or "Urate" Layer. The reflector layer differs from the photogenic layer in a number of respects. First, the cytoplasm of its cells usually fails to stain with ordinary acid dyes such as eosin, so that in conventional histological preparations the layer is clear, in contrast to the heavily stained photogenic layer. Second, its cells are packed with small, highly refractile particles, probably birefringent and often described as "crystals" or "sphaerocrystals," but also reported by some to have a rounded outline. In sections of fresh tissue, these particles render the reflector layer opaque by transmitted light, in contrast to the translucent photogenic layer, and white by reflected light, in contrast to the dark photogenic layer. In balsam preparations, on the other hand, the "crystals" are mainly dissolved out by the dilute alcohols and leave the reflector layer nearly hyaline. Other differences, such as solubility in various reagents, have been described (Kölliker, Schultze, Wielowiejski, Bongardt, Townsend; Hasama, 1942a). End-cells have been reported from the reflector layer by Geipel, but according to Lund, Bongardt, and Emery, are very rare or absent.

The two-layered nature of the light organ was first recognized by Kölliker, who described chemical tests purporting to show that the material in the dorsal (reflector) layer is ammonium urate. While there is no reason to doubt this conclusion, and although the work has been repeated a number of times with only minor discrepancies (Owsjannikow, 1868; Tozzetti, 1870; Heinemann, 1872 and 1873; Bongardt; Lund) it has not been checked by modern analytical techniques. In assessing all the work on the chemical composition of the luminous organ or its layers, it must be kept in mind that the two layers have not been separated before testing, so that even microchemical color reactions *in situ* are by no means devoid of the possibility of contamination from the contiguous layer, or indeed from other viscera.

No agreement exists as to the function or functions of the reflector layer. Most workers have accepted, explicitly or implicitly, Kölliker's idea that it serves as a physical reflector to increase the amount of light emitted. No experimental work has been done on this point, however, and in view of the granular type of cytoplasm, I doubt whether the reflecting efficiency of the layer can be very high. In addition, much light would be absorbed in passing back and forth through the photogenic layer. The somewhat similar notion (McDermott and Crane) that the layer may act as a screen to protect the deeper-lying tissues from the light seems likewise to be questionable, because of the lack of evidence or expectation that the light produced in the photogenic cells is injurious.

The presence of urate in the reflector layer has led to a flood of mostly gratuitous speculations on possible metabolic connections between photogenic and reflector layers, even including a contention that the urate granules themselves are the true source of light (Weitlaner, 1909). Although a membrane between photogenic and reflector layers has been described (Tozzetti), the great majority of accounts show the two layers abutting directly (or even with a contact line so irregular that isolated photogenic cells project into indentations in the reflector layer and *vice versa*), so that a direct passage of particulate material between them is at least theoretically possible.

One idea which had considerable vogue provides that the cells of the photogenic layer, as they get "used up" and filled with the waste products of their activity, are transferred to the reflector layer (Schultze, Wielowiejski,* Dubois; Gerretsen, 1922; denied, however, by Emery and by Lund). Such a process is difficult to visualize for any but the photogenic cells already contiguous to the reflector layer, and would be equivalent simply to shifting the boundary between the layers rather than to actual cell movement. This would require, first, that the photogenic cells closest to the reflector layer, in order to be the first to transform, indulge in the most intense activity, and second, that with time the reflector layer should increase in thickness at the expense of the photogenic layer. Support for either of these points is lacking in most recent investigations, although either proof or disproof would be very hard to obtain, if for no other reason than the great difficulty in assessing and comparing total light emissions. As a matter of fact, "transition" cells† between the two layers have been described (Kölliker; Hasama, 1942a‡; see also FIGURE 23), and Weitlaner (1911) reported an increase in the amount of urate in old as compared with young individuals. On the other hand, Emery found no difference between young and old animals, and Townsend, Lund, and Geipel saw no seasonal change in thickness of either layer. Heinemann (1872) also found no change in four weeks in *Pyrophorus*. Okada (1935a) reported a decrease in thickness of the photogenic layer with age, without change in the thickness of the reflector layer. Hasama, in several species, found no difference in size or distribution of photogenic granules from fireflies long in darkness compared with fireflies after a long period of flashing. Moreover, the presence of transition cells has been denied specifically by several writers (*e.g.*, Lund, Geipel). In view of the considerable variability in layer thickness in different individuals, and particularly in different regions of the same organ (even if care is taken to use only sections exactly perpendicular to the surface), all such work is suspect until confirmed on numbers of specimens adequate to demonstrate statistically significant differences.

A somewhat similar idea is that the urate granules represent waste products of the oxidation of the photogenic granules in the light cells, which have been transferred by an undescribed process to the reflector layer and there stored (Kölliker, Tozzetti, Lund). Lund has championed this view strongly. His claim that the "waste product" deposited in the dorsal layer is "allied to or identical with some of the split products of nucleic acid" is interesting in view of the recent demonstration that firefly light organs contain a relatively high proportion of flavin-adenine dinucleotide (Ball and Ramsdell, 1944).* However, not only is there no decisive evidence of an actual transfer of material between layers, but, as Okada (1935a) has pointed out, there are difficulties in explaining how a waste material, which (according to Lund) crystallizes in the photogenic cells, comes to be redissolved, transferred (there is no blood circulation directly between the two layers), and recrystallized in the cells of the urate layer. Furthermore, Lund's "striking and conclusive" proof of a "direct relation and actual tracing of products of decomposition resulting from photogenesis from their place of origin in the photogenic cells into the dorsal layer cells" consists of no more than the following observation: When a cross-section of an entire photogenic organ is viewed by reflected light, the dorsal (reflector) layer was seen to be packed with a dense white mass (crystalline deposit), and in the ventral layer similar ("same") material was seen in the most peripheral cytoplasm and sometimes around the nuclei of the photogenic cells. Small amounts of this material were also observed by Lund in other viscera, and other investigators have reported that "urate" occurs in the fat body in considerable concentration. Lund also claimed that he found "different amounts of the accumulated products of katabolism in different species and different specimens of the same species," and that "the degree of filling of the dorsal layer cells also corresponds to the amounts of the deposit upon and in the photogenic cells." These claims are in direct opposition to the observations of several workers, particularly Emery, and Geipel. By implication, Lund regarded these differences as progressive stages in a process starting with no crystalline waste in the photogenic cells at the beginning of adult life, and culminating in a heavy deposit in aged specimens.

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* **Wielowiejski**, in a later paper, came to the opposite conclusion in regard to American fireflies.

† Not to be confused with the term, transition cell, used by **Holmgren** (1895) and **Townsend** as synonymous for end-cell.

‡ **Bongardt** saw cells intermediate in character, but not at the boundary between the layers.

Lund's theory implies that at any moment the photogenic cells of a given firefly would present a cytoplasmic appearance directly related to the total amount of light which had been produced. Since such an assumption is prerequisite for all ideas of photogenic-urate transformation, it is germane to point out, first, that with a number of techniques a considerable cytoplasmic variation is apparent not only between the photogenic cells of the same individual but between contiguous cells, and second, that the finding of differences does not necessarily justify arranging them artificially into an irreversible temporal sequence. It seems more reasonable to assume that the photogenic cells go through individual, and probably asynchronous, cycles of activity which are repeated a number of times during the functional life of the photogenic layer. Such a view would agree with what occurs in many gland cells. At present, we can only speculate on what this "activity" is. However, the schemes of enzymatic oxidation and resynthesis of luciferin which have been developed in recent years furnish a reasonable pattern (see summary in Johnson *et al.,* 1945). It is true that the cytological differences between cells of the same organ could be explained by assuming that all the cells are in a stable equilibrium of oxidation (photogeny) and synthesis, but that the point of equilibrium (intensity of activity) differs in different individual cells and is reflected in their appearance. Critical information bearing on these various possibilities would be of great importance in questions of chemistry and physiology of the photogenic process and the mechanism of control, but none exists aside from scattered observations of spotty or localized glowing of the photogenic organ. These, however, indicate the possibility of differences in the responses of different cells.

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* It is also interesting in view of **McElroy & Ballentine's** (1944) claim that phosphate is liberated during the *in vitro* luminescent reaction of *Cypridina*. **McDermott** (1915) attempted to test **Lund's** hypothesis by comparing the soluble phosphorus and nitrogen content of a solution of dried and powdered firefly organ which was allowed to luminesce in the presence of peroxide, with that of one prevented from luminescing. He found slightly less of both phosphorus and nitrogen in the solution which had luminesced, and concluded that **Lund's** theory was unsound. It is not clear, however, that a change in phosphorus and nitrogen should have been anticipated in **McDermott's** experiment.

The fact that organs of Types 1 and 2 operate without a reflector layer could be construed as indicating that it cannot have any essential connection with photogeny, although Wielowiejski argued that, since these organs are characteristically very small, their wastes could diffuse away directly.

Recent biochemical schemes (*e.g.,* McElroy and Ballentine, 1944), provide for the resynthesis of at least part of the oxidized luciferin. If this occurs, and if the necessary energy is supplied by reactions having gaseous or easily diffusible end-products, there need be no accumulation of organic waste products in the photogenic cell and no necessity for providing elaborate mechanisms for their removal. There is also the possibility that reactions which are not reversible *in vitro* (*e.g.,* the luminescent oxidation of *Cypridina* luciferin) are reversible *in vivo,* making possible an even greater conservation of material by cyclic reutilization.

On the whole, therefore, there has been no clear demonstration of what the function or functions of the reflector layer are, although the large bulk of the tissue, its close association with the photogenic layer, and its regular presence in many fireflies suggest that it must be of utility to the organism.

The Tracheal End-Cell. The end-cell has been the subject of much detailed study, since its strategic position at the beginning of the tracheoles at once suggests that it may be concerned in controlling the oxygen which, presumably, passes through the tracheoles into the photogenic cells. The end-cell is by no means a typical cell. Ordinary techniques often fail to reveal much more than its nucleus. The cytoplasm is best demonstrated by subjecting the intact animal to osmic acid vapor. This apparently diffuses unaffected through most of the tracheal system, but escapes into the cytoplasm in the region of the end-cell, where it is reduced to the black suboxide or metallic form. Progressively heavier doses delineate progressively more of the cytoplasm and processes of the end-cell, and sometimes the tracheoles. The number of processes present usually corresponds to the number of tracheoles given off, where the two are distinguishable. It varies from two to seven in different species, though it is usually rather constant intraspecifically.

It is sometimes not clear how the tracheoles originate within the end-cell. Geipel shows the tracheal twig terminating blindly within the end-cell, without any tracheoles being present (FIGURE 3). However, the most popular idea involves a continuous tubular connection, within the end-cell, between the narrowed tip of the tracheal twig (or a common tracheole issuing from this tip), and the tracheoles (see FIGURES 2, 11, and 13; also the drawings of Wielowiejski). According to Bongardt, and Dahlgren, there is a darkly staining annular swelling ("rounded body" or "ampulla") around the common tracheole (FIGURE 11, "S"), for which sphincter properties have been postulated (Dahlgren; Creighton, 1926; Snell, 1932; Alexander, 1943). Little differentiation has been observed in the cytoplasm of the end-cell, except by Dahlgren, who described "contractile radial fibers" (FIGURE 11). This observation is considered in detail in the last section of the present paper.

Lund seems to have regarded the permeability to osmic vapor, and the ability to bring about its reduction, as specific and exclusive properties of the tracheal end-cell and the tracheolar wall or membrane. On the basis of the temperature lability of the osmic reduction effect, he even ascribed it to the presence of a specific "reductase", which he assumed also to perform an analogous function in transferring oxygen in luminescence.[*] Wielowiejski had a somewhat similar idea. However, it has long been known that, with heavy doses, the area of reduced osmium spreads beyond the end-cell into the cytoplasm of the contiguous photogenic cells. Moreover, it was shown by Wielowiejski that, in Type 3 organs (which lack end-cells, and in which the tracheoles do not all originate at one point), the tracheoles are nevertheless permeable to osmic acid vapor and the surrounding photogenic cytoplasm is able to accomplish its reduction.[†] This was also shown clearly in *Pyrophorus* by Geipel.

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[*] **Remy** (1925) found (not in fireflies) that the tracheal wall recolorizes indigo-white and, hence, has an "oxidative power." It is not clear, however, why this effect is not due merely to the proximity of gaseous oxygen. Aside from luciferase, no enzymes have been separated from photogenic tissue. Among other enzymes which have been postulated is Gerretsen's "photogenase", which presides over the formation of luciferin. Some suggestion of catalase activity is contained in **Burge's** (1916) claim that fresh firefly tissue liberated more oxygen from hydrogen peroxide than did an equal weight of bee tissue, and that the "luminous part" of a firefly liberated more than the non-luminous. The work, however, was poorly controlled.

[†] **Wielowiejski** distinguished clearly and categorically between Type 3 organs with their tree-like tracheal branching (such as those of *Lampyris* or the minute "tuberculate" organ of the female of *Lamprorhiza splendidula*) and the Type 5 organs with end-cells of *Lamprorhiza splendidula*. He did point out, however, that sometimes in Type 3 organs the point of origin of tracheoles, especially if more than one tracheole is involved, may simulate an end-cell due to the reduction of osmium there. This is particularly true since the thin tracheal epithelium often fans out at such loci. **Geipel** apparently observed similar structures in the reflector layer of *Photinus marginellatus*, which he interpreted as a different type of end-cell. **Bongardt**, working with the same materials as **Wielowiejski**, apparently duplicated the latter's observations very closely, but his presentation and discussion are so incredibly incoherent and contradictory (culminating on his page 25) that I found it impossible to make out whether he even believed in the existence of end-cells at all.

One of the least clear features of the end-cell is its external surface. This subject, though rather esoteric, is considered here in some detail, because it may help to clarify a large mass of confused literature and because it bears on certain properties of the end-cell and tracheoles which are of physiological interest. In macerated preparations (FIGURES 27, 37, and 40), the end-cells maintain their integrities as separate masses of protoplasm. However, their apparent limits seem often to depend on the degree of osmic impregnation (*e.g.,* Schultze, Townsend, Lund) and often no sharp membrane can be made out in either fixed or fresh preparations. This fact is illustrated in Townsend's and Dahlgren's figures, and I have observed it in most of the species of *Photinus* which I have studied (FIGURES 31, 37, 39, and 40). Emery was even led to consider all the contents of the cylinder as a sort of syncytium, and both Emery and Townsend regarded the end-cells as artifacts. It is clear from their figures, however, that they were dealing with the same structures which we now call end-cells. On the other hand, many investigators figure the end-cells as with a definite boundary, although they are vague about its nature (*e.g.,* FIGURES 2 and 3). Part of the confusion results from the fact that the tracheal end-cell is believed to be a derivative of the thin tracheal epithelium which covers all the tracheae, including the lateral twigs in the cylinder (Lund; Williams; Hess, 1922). Wielowiejski and Emery, for example, conceive of this epithelium as stretched over the flat fan-like furcation point of the tracheoles, like the web of a duck's foot, thus forming the "membrane" of the end-cell. This membrane, in turn, is produced as an "end-cell process", and invests each tracheole individually throughout its course (or, according to Bongardt and Williams, part of its course). Over most of this distance, the epithelium is indistinguishable from the tracheolar wall proper, because of its extreme thinness. On the other hand, in regions where the two can be separated visually, they can be differentiated also by the use of strong alkali, which dissolves the former, leaving the chitinous (*sic*) tracheole.

Other descriptions of end-cells differ in several respects from that of Wielowiejski. According to Bongardt, the end-cell processes accompany the tracheoles for long distances in the photogenic tissue, branch and anastomose richly on the surfaces of the cells, but eventually terminate and leave the individual unbranched tracheoles to run alone farther into the tissue. Geipel, on the other hand, seems to find no typical tracheoles at all in *Photinus marginellatus,* but only long, hollow, tapering end-cell processes, like the roots of a molar tooth, which embrace the photogenic cells (see also FIGURE 10).* Likewise, most of the species studied by Hasama have tapering end-cell processes. Here, however, a tracheole runs in each process up to, but not beyond, its tip. My observations on *Photuris,* which has end-cells apparently very similar to those of *Photinus*

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* The tracheoles shown within the end-cell and processes of **Dahlgren's** reproduction (his FIGURE 19) of **Geipel's** figure (here reproduced as FIGURE 3) are not in **Geipel's** original.

marginellatus, lead me to believe that Geipel overlooked all, and Hasama part, of the tracheoles. In *Photuris,* these extraordinarily delicate tubules can be seen running from the tracheal twig into each end-cell process, and then issuing from the distal end of each process and proceeding farther into the photogenic tissue. This is visible in preparations made by several standard methods, and thus is unrelated to the vagaries of osmic acid penetration.

In summary, it seems justifiable to conclude that all the types of end-cells discussed are built on the same fundamental plan of multiple tracheolar branching from a tracheal twig.

There seem to be two main types: the *Photinus* type (with some exceptions), in which the end-cell is strictly limited to the cylinder, has an indistinct "cell membrane", and gives off usually two tracheoles of uniform diameter (FIGURES 11, 22, 26, 31, and 38); and the *Photuris-Lamprorhiza-Photinus marginellatus* type, in which the end-cell projects into the photogenic tissue, has a definite "cell-membrane", and gives off usually four to seven apparently tapering processes which accompany the tracheoles for varying distances into the photogenic tissue (FIGURES 6, 10, 14, and 32). There are numerous additional minor discrepancies between the various accounts of end-cells. Some of these are doubtless *bona fide* differences in material. However, there is probably considerable justification for attributing much of the contradiction to inferior optics, since the homogeneous immersion lens was not in general use until about 1885. This is the more reasonable in that the details in question are near the limits of microscopic resolution.

The Photogenic Cytoplasm. The strikingly granular contents of the photogenic cells have attracted the attention of numerous workers. The granules are minute, usually spherical and densely packed, and take acid or plasma dyes. Dahlgren claims that tissues fixed in boiling fixatives show that the granules are spherical in the male, rod-shaped in the female (FIGURE 11). Hasama (1942a) could not confirm the distinction in *Pyrocoelia rufa,* but says that Okada found about half the females in *P. consanguineus* differing from the males in the manner claimed by Dahlgren. In *Photinus pallens,* I found that granule shape is more or less constant in a given individual and may differ in different individuals, but is not characteristic of either sex.

Dubois regarded the photogenic granules ("vacuolides") as self-perpetuating entities comparable to mitochondria, and a similar concept is implied in Dahlgren and Kepner's term "photochondria". Vonwiller (1921) claimed that the granules stained like mitochondria, but Takagi (1934) showed that mitochondria could be demonstrated independently of the granules in *Luciola cruciata.* Moreover, Dubois was unable to culture granules isolated from several organisms.

Kuhnt (1907) suggested, by analogy with leguminous root-nodules, that the firefly light organ contains symbiotic bacteria. Pierantoni (1914), influenced by his studies on the symbiotic "mycetome" organs of Hemiptera, claimed to have cultured two kinds of bacteria from both the photogenic organ and the egg of *Lampyris*. These "bacteria" were described as distinct from the photogenic granules. Their figured appearance strongly suggests that they are mitochondria. Pierantoni did not make single-cell isolation cultures, and his evidence for the transmission of the "bacteria" by way of the egg is unconvincing. Furthermore, his cultures were not luminous. This is not necessarily a decisive objection in view of the known dependence of bacterial metabolism on culture conditions. Also, similar results have been obtained in various organisms, particularly fish, where the evidence for bacterial symbioticism is much more sound than it is in fireflies (see Harvey, 1940, pages 30 to 36). Vogel (1922) and Hasama (1942a) were unsuccessful in culturing anything from the light organs of fireflies, although Hasama's failure may have been influenced by his choice of 37° C. as an incubation temperature. Buchner originally (1914) supported Pierantoni's thesis, though he never, himself, worked on Lampyrids. In the second edition of Buchner's book (1930), however, the matter is left undecided.

Harvey and Hall (1929) demonstrated that the development and functioning of the adult light organ in *Photuris pennsylvanica* is unaffected by ablation of the larval organs. They concluded, therefore, that bacteria are not concerned with luminescence. They acknowledged, however, the possibility that bacteria in a non-luminous phase might have existed in other parts of the body and have contributed to the formation of the adult organ.

On the whole, although the photogenic granules and other cytoplasmic inclusions often resemble bacteria in form, size and staining, and although some organisms apparently do have organs (even luminous organs) which contain symbiotic bacteria, the evidence in regard to fireflies is so weak that a definite conclusion would be wholly gratuitous.

A number of workers (*e.g.*, Williams) have described the staining reactions of the photogenic granules with a variety of dyes. Since, however, most such reactions are highly unspecific in a chemical sense, the most that can be concluded is that the granules probably contain protein. The granules have been identified as "the" photogenic material (Dubois; Dahlgren and Kepner, 1908; Lund, McDermott, Dahlgren, Williams, etc.), or even specifically as "luciferase", "photogenin" or "luciferin", with very tenuous justification except that granules of some sort seem to be associated with light-production in all known animals. It is outside the scope of this paper to go into the chemistry of the photogenic layer in detail, but it is obviously relevant to mention that analyses of *whole* organs by Dubois, Harvey, Gerretsen (1922), McDermott, and others, have shown that luciferase and luciferin are present (or, at least, that two extracts can be prepared which luminesce when mixed). These are universally assumed to be localized in the photogenic layer. Considering the certainty that the photogenic layer contains a number of compounds of varying complexity, and in view of the probability of contamination already mentioned, it is not surprising that earlier analyses of the material of the "photogenic layer" yielded identifications as diverse as "albuminous" (Kölliker), "lecithin-like" (Lund), and "phosphatide with an aliphatic radical" (McDermott, 1911b).

Another interesting feature in the cytoplasm of Type 6 photogenic layers is the striking "differentiated zone" which surrounds the cylinders, (figures by Lund; Hess, 1922; Dahlgren; Williams; and Okada, 1935b). This zone is formed by a part of the peripheral cytoplasm of the photogenic cells in which the so-called photogenic granules are lacking, and only an extremely fine-grained and compact-looking cytoplasm can be seen (FIGURES 10, 11, 22, 26, and 31). The zone is of varying thickness in different species and in some has apparently not been recognized. That it differs chemically or physically from the rest of the cytoplasm, is indicated by its different response to a number of dyes. It is traversed, of course, by the tracheoles or by the end-cell processes as they pass from the cylinder into the luminescent tissue. Thus it is, by inference, the region where photogenic material might first come in contact with oxygen. According to most workers, this differentiated region is not found between different photogenic cells but only between the cells and the cylinder, or along the dorsal and ventral surfaces of the photogenic layer (FIGURE 26). In some of my preparations, however, it apparently also forms a very thin layer along the internal faces of the photogenic cells. The zone thus envelops the photogenic cells completely and "insulates" their interior cytoplasm. Possibly with this thought in mind, Dahlgren made the stimulating but unsupported proposal that this zone is impermeable to oxygen and serves as a protection against the "entrance of any oxygen that might come into the cells except through the tracheal capillaries." Aside from the difficulty of visualizing the mechanism of this impermeability, many firefly photogenic organs are apparently not equipped with such a protective layer and yet do not show uncontrolled luminescence. There may be some merit in the general concept, however, since Wigglesworth (1930), and others have shown that the walls of even large tracheae are permeable to oxygen. This suggests, in view of the low partial pressures of oxygen necessary to support luminescence (see page 442), that the quantity dissolved in the general body fluids might be sufficient to support luminescence, if there were no insulation. Also, as suggested on page 424, the differentiated layer might possibly have some bearing on observed differences in types of luminous emission (see also page 449).

The final peculiarity of the photogenic cytoplasm here considered is the unobtrusiveness of its limiting membrane. To be sure, the presence of sharp cell boundaries is described, or implied, by numerous workers (*e.g.*, FIGURES 6, 9, and 10; see also Hasama, 1944b), but in other instances tribute is paid to the fact that often no membrane of any sort has been observed (FIGURES 7, 11, 14, and 23). Lund, in fact, frankly regards the photogenic cytoplasm as a syncytium. This may be so in some species, but I hope to show later that in *Photuris pennsylvanica,* to which Lund referred particularly, cell membranes can be demonstrated. One reason why the membranes are difficult to find in Type 6 organs, is that they are concealed or obscured by the tracheoles. For example, in a horizontal section of the photogenic layer, the "lines" delineating the rosette pattern ordinarily appear single, and it is virtually impossible to decide, even with progressive focusing, whether one is looking at a tracheole or at a cell membrane in edge view (FIGURE 22). Even a double line would not be conclusive, since "the" membrane separating two photogenic cells is presumably double. Likewise, in a cross-section of the organ, some membranes are in face or quartered view, and hence invisible. Usually, it is only when a section is chosen so that an edge view of a membrane and a cross-sectional view of tracheoles are seen simultaneously, that one can distinguish the two with certainty. Figures of such views have been published by Bongardt, Townsend (and Geipel, for the end-cell processes), and examples from *Photuris pennsylvanica* and *Photinus pallens* are presented in FIGURES 35 and 36. In horizontal sections of the organ, cross-sections of tracheoles can usually only be seen close to the ventral (outer) surface, since it is only here that many of the tracheoles run vertically (FIGURE 36).

The Tracheoles. The origin of the tracheoles and their general arrangement should be clear from the discussions and figures already presented. Measurements of the dimensions of tracheoles (or, indeed, of any part of the luminous organ) are rare. The only figures for tracheolar diameter are Wielowiejski's and Lund's 1.3 and 1.1 microns, respectively, and for length, Townsend's value of 20 to 60 microns (estimated from her figures on the distances between cylinders in *Photinus marginellus*). The quoted dimensions are of the same order of magnitude as the averages obtained by the writer, but there are wide differences between different species, and the diameter measurements are probably subject to large errors as well as to variations caused by different techniques of preparation. In *Photuris,* the tracheoles issuing from the ends of the end-cell processes appear to be much less than a micron in diameter. In organs of Types 2, 3, and 5, where anastomosis probably does not occur, the tracheoles are much longer and by no means uniform in diameter at their origins. Moreover, they taper, so that diameters approximating or exceeding the resolving power of the microscope may be reached near their distal ends.

One hotly debated morphological point, which has physiological implications also, is whether the tracheoles actually penetrate the cytoplasm of the photogenic cells or are exclusively extracellular in their courses. Heinemann (1872) reported that the tracheoles impale the photogenic cells in *Pyrophorus,* though Robin and Laboulbène (1873), and Geipel described them as applied to the cell faces. Lund strongly espoused intracellular penetration in the several lampyrids he studied. However, the majority of workers on lampyrids have reported that the tracheoles (or end-cell processes) run only between or on the outside surfaces of the photogenic cells (Wielowiejski; Emery; Watasé, 1895; Bongardt; Townsend; Geipel; Dahlgren; Williams; Okada, 1935b; and Hasama). Both intracellular and extracellular tracheoles have been reported in other kinds of insects (Wigglesworth, 1930 and 1939). Lund's stand was primarily based on finding tracheoles close to nuclei in the same focal plane, mainly in *Photuris pennsylvanica.* I think this observation was a misinterpretation, due to the fact that in this species, the limits of the cells are poorly delineated and the cells are often irregular in shape, overlapping, and scarcely wider than the nucleus (FIGURE 35). At any rate, I have found no case of intracellular penetration of tracheoles in any of some twenty Jamaican and American species of *Photinus* and *Photuris.* Perhaps the most convincing evidence comes from the type of preparation already referred to, in which cell membranes and cross-sections of tracheoles both appear (FIGURES 35 and 36). Another type of evidence is seen in surface views of freshly extirpated light organs which have been dried sufficiently for air to enter the tracheoles. Here, the tracheoles follow a course similar to that figured by Townsend (FIGURE 13), which corresponds exactly to the intercellular interfaces seen typically in the rosette pattern of horizontal sections (FIGURES 29 and 38).

Another disputed subject is whether the tracheoles end free or anastomose with other tracheoles. This has physiological implications, since anastomosis might permit gas flow through the tracheoles, whereas transport to free endings would almost certainly be by diffusion. Anastomoses have not been reported in organs of Types 2 and 3. In *Lampyris* and *Lamprorhiza* the existence of "loops" was claimed by Kölliker, and denied by Schultze, but since tracheoles were unknown at that time, it is not clear what was meant. However, Wielowiejski found anastomoses only occasionally, and Bongardt states that they do not occur.* Five of the apparently Type 5 species discussed in Hasama's papers are likewise of the *Lampyris* type, since the end-cell processes (which are said to wholly contain the tracheoles) do not anastomose (*Pyrocoelia rufa* and *P. analis; Luciola lateralis, L. cruciata* and *L. gorhami*). In most Type 6 organs, profuse tracheolar anastomoses between contiguous cylinders are described (McDermott and Crane, Lund, Townsend, Williams, and Buck, 1942). Emery saw no anastomoses in *Luciola italica,* though his FIGURE 7 closely resembles most Type 6 organs (*e.g.,* FIGURES 13, 29, and 39). Tozzetti apparently saw some anastomosis *within* the cylinder in *L. italica,* although his descriptions and figures are not entirely clear. Okada (1935b), however, shows the end-cell processes ending free (FIGURE 10). On the whole, there appears to be a systematic difference between Types 5 and 6 in regard to anastomosis of tracheoles. This should be kept in mind during the discussion of flashing behavior in the two types.

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* However, Bongardt described the tracheoles as running in the end-cell processes, and the latter as branching and anastomosing richly!

[*Note Added in Proof.* Dr. A. Glenn Richards recently made a number of electron micrographs of tracheoles from fresh light organs from males of *Photinus pyralis* which I supplied. The prints (3800 X) show isolated tracheoles of very uniform diameter (about 0.25 μ) and up to 25 μ long. In accordance with Richards's findings in many other insects, the tracheoles show clear spiral thickenings throughout, and appear to end blindly, without anastomosis. I have some doubts as to whether the tracheoles are actually those originating from end-cells in cylinders, because in several instances they appear to arise in groups of three, whereas in histological preparations the number is invariably two. Moreover, the advantages devolving from the increased resolution of electron optics are somewhat offset by the necessarily drastic method of preparation (maceration in water or weak alkali, teasing, drying *in vacuo,* and exposure to electron beams). Nevertheless, Richards's pictures caution against any dogmatic insistence on the existence of tracheolar anastomosis.]

Several writers have quoted Schultze as having shown the tubular nature of the tracheoles by gold chloride impregnation, forgetting that he never saw the true tracheoles. Wielowiejski, however, demonstrated the lumen by infiltration with dyed soap. It is now generally agreed both that they are hollow, and that the tracheolar wall is exceedingly thin. Both facts are illustrated in those figures of Bongardt and of Townsend which show cross-sections of tracheoles, and in FIGURE 36.

Though all agree that the tracheoles are hollow, there is less unanimity on what they contain, at least in life. Schultze, Tozzetti, Emery, and Lund claimed that in freshly dissected photogenic tissue the "tracheoles" (in some cases the finer tracheae must be meant) are nearly or entirely invisible, because they contain liquid. Tozzetti suggested that the tracheoles might carry part of the blood circulation. Wielowiejski and Townsend found that glycerin enters the air-filled tracheoles of a dried organ from the inside out (*i.e.,* distal-proximal direction), from which they concluded—irrelevantly, it seems to me—that the tracheoles are air-filled in life. No observations have been made on live fireflies, nor are any to be expected, in view of the thickness and delicacy of the photogenic tissue. On the other hand, in insects such as fleas, and mealworm and mosquite larvae, which, admittedly, have different anatomies, the situation in life is apparently quite variable and changes under various conditions (Wigglesworth, 1939). It is clear that, in some instances, the tracheoles are normally air-filled down to diameters of 0.5 micron and, since this is the order of magnitude of firefly tracheoles, there seems to be no reason why they might not be air-filled. As a matter of fact, unless the tracheoles were gas-filled throughout, intracellular penetration of tracheoles would confer no advantage, since the distribution of oxygen in the photogenic cytoplasm (if such is the purpose of the tracheoles) must ultimately occur by aqueous diffusion, which could only be hampered by a tracheolar wall. This matter will be further discussed in the section on physiology.

The Nerves. The least-known major anatomical feature of the photogenic organ is its nerve supply. In spite of contrary statements in the literature, I have not found the ganglia supplying the photogenic segments to be disproportionately large in comparison with those of similar non-luminous insects. Kölliker and Schultze were the first of several to report having seen nerves in the photogenic organ. With rare exceptions, however, these structures were demonstrated by reagents and techniques which are not recognized today as having diagnostic value in the identification of nervous tissue. Therefore, since even the standard neurological techniques are too often unpredictable, the possibility of artifact deserves serious consideration. This is the more pertinent in view of the great difficulty reported in demonstrating the "nerves" and especially in distinguishing their fine branches from tracheoles. Also, some very atypical structures have been described, as, *e.g.*, the "knobbed" and "multinucleate" fibers of Wielowiejski and Bongardt.* Wielowiejski devoted several pages to a description of connective tissue fibers (in non-photogenic tissue) which previous workers had misidentified as nerves, indicating that the techniques then in use were by no means reliable.

However, taking the reports at face value, the nerves generally follow the tracheal system rather closely (though Wielowiejski denies this) and are distributed in roughly the same fashion. There is much disagreement as to the ultimate terminations of the nerves, which are, of course, the regions of greatest interest. Tozzetti and Emery found no connection between nerves and any sort of cells in *Luciola.* Kölliker and Schultze could not trace the finer nerves to their ends in *Lamprorhiza,* though Schultze thought it likely that they innervated the photogenic cells. Wielowiejski found direct connections between nerves and the surfaces of photogenic cells in *Lamprorhiza,* as did Owsjannikow (1868) in *Lampyris.* Owsjannikow even described the nerve as penetrating to the nucleus, though Wielowiejski decried this idea. Geipel and Hasama (1942a) reported that in *Photinus marginellatus* and *Pyrocoelia rufa,* respectively, the finest nerves connect directly with the end-cells (to the nucleus, according to Geipel), and in *Lamprorhiza* and *Lampyris,* Bongardt found them connected with the exterior of both end-cells and photogenic cells.

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* **Lindemann** (1868) described "nerves" running to the "light-balls" in *Lampyris,* but his descriptions and conclusions are so bizarre that I can only conclude that he had mistaken some other tissue for the photogenic organ.

There is, therefore, no general agreement on the details of the nervous supply of the photogenic organ. In my opinion, a really convincing answer to this problem will require a full investigation which is devoted to this one point and makes use of modern neurological techniques.

A Possible Ultra-tracheolar Network. In preparations made by silver nitrate impregnation, the tracheoles are heavily outlined in black by precipitated silver. In cross-section, they appear as little black circles strung on dotted black lines which are formed by a light deposit of silver on the photogenic cell membranes (FIGURE 36). Wherever the plane between two tracheoles (which means the plane of a cell membrane) is horizontal or nearly so (that is, parallel with the stage of the microscope), the tracheoles are seen to be knit together by a close-meshed network (FIGURES 33, and 34). This remarkable structure is only visible here and there at any one focus in a given field, in agreement with the rarity of instances where a flat region of cell membrane happens to lie parallel with the surface of the section, but it is often possible to trace the network over a relatively large area by careful focusing. The individual strands of the network are far too slender (of the order of 0.1-0.2 microns) to make it possible to say whether or not they are tubular. I have found them in *Photuris pennsylvanica* and *P. jamaicensis,* and in *Photinus pyralis* and *P. pallens.*

This network could be interpreted as a mesh of extraordinarily fine tubules binding the tracheoles together, in a manner analogous to the capillary networks between arterioles and venules. This might make a more satisfying picture, physiologically, than tracheoles alone, because such a network might permit quicker and more uniform distribution of oxygen to the photogenic cell, and thus reduce the partial pressure of oxygen necessary to support luminescence. However, in spite of the sharpness, relative orderliness, and wide distribution of this structure, I am not insisting on the above interpretation for the present, because metallic impregnation is such a notoriously capricious technique that we must reckon with the possibility of an artifact. Furthermore, in maceration preparations, and in those prepared with caustic, where the tracheoles are relatively free from other tissue, they do not behave as if held together by a network, nor do they show a rough outline such as might be left if the network had been torn off.

On the other hand, "ultra-tracheolar" offshoots of tracheoles have been figured from other insects, though not in a network (Wigglesworth, 1939, FIGURE 164).* The reason that the network appears only with the

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*The networks described by **Wistinghausen** (1895) and **Holmgren** are larger than those discussed here by a factor of ten. Other supposed tracheolar networks have been reported but have been shown to be present also in vertebrate tissue (see **Wigglesworth,** 1931).

silver nitrate technique is undoubtedly that the "tubules" are so delicate that they would be invisible without a completely opaque coating. This probably explains, also, why they have not been reported previously, since silver nitrate has been used on firefly organs only very rarely (Owsjannikow, 1868, Tozzetti; Geipel).

PHYSIOLOGICAL ASPECTS OF LUMINESCENCE

Introduction. It was formerly customary for writers on fireflies to include a section or appendix on "physiology." Unfortunately, far too many of these were concerned only with a perfunctory and uncritical exposure of fireflies to various common laboratory reagents. Some of the early work, nevertheless, led to fundamental conclusions in spite of the understandable crudeness of the experiments. In considering modern physiological theories, we shall make extensive use of two of these general findings: the respective influences of the nervous system and of oxygen on luminescence.

The remaining "physiological" literature falls into three classes. The first deals with the actual nature of bioluminescence. These old arguments and experiments over whether fireflies light by means of phosphorus, crystallization, phosphorescence, etc., are interesting historically, but of no relevance to the present discussion since it has been generally agreed for over fifty years that animal light involves an enzymatic oxidation of an organic substrate. The second class of literature concerns the question of whether or not the light is a vital phenomenon, and again this can be dismissed, for no one since Pflüger* (1875) and Bellesme (1880) has doubted that the actual luminescent reaction can occur in the absence of living protoplasm, or even *in vitro*. Finally, there is a huge mass of heterogeneous and unsystematized work which concerns almost every aspect of bioluminescence. Included in this category, for example, are the work on the effects of temperature, the papers on the spectral character of the light (for review, see Buck, 1941, and Grinfeld, 1944), and particularly the reports on the effects of literally hundreds of gases, vapors, acids, bases, salts, poisons, drugs, solvents, excretions, enzymes, etc., on intact fireflies and on isolated organs. All this work suffers from the fundamental ambiguity that it is uncertain whether the agent is acting directly on the actual luminescent reaction or upon a biological mechanism controlling it, as, for example, the nervous system. Furthermore, much of this research is vitiated by failure to specify concentrations, particularly of gases and vapors, and because of the use of single, or only very few, fireflies for each reagent tested. Obviously, we shall be able to utilize only small and isolated fragments from this literature in pursuing the problem outlined in the general introduction. Some of the remaining information, however, is of potential physiological use, though at present one can draw only the most obvious sorts of conclusions, such as that strong protein precipitants extinguish luminescence. The data have further value in showing that one cannot possibly regard the depression or stimulation of luminescence by a given agent as a specific effect, and that one cannot safely argue by analogy, in the sense of saying, for example, that because a capillary dilator increases luminescence, it does so by dilating the tracheoles.

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* The facts usually cited as evidence that light-production can be independent of life are the following: (1) Fireflies may still be glowing several days after their apparent deaths. (2) Fireflies which have been quickly dried and kept in absence of air, may glow when moistened, even after several years (*e.g.*, **McDermott**, 1915). **Pflüger** (p. 287) countered these two arguments with allusions, respectively, to the sustained irritability of extirpated (surviving) frog muscle, and the viability of cysts of rotifers, etc. Most workers, however, consider these arguments invalid.

Normal Types of Light-Emission. The difficulties in analyzing the various types of luminescence seen in different kinds of fireflies are increased by the fact that many workers have not distinguished between luminescent behavior in the field and that under laboratory conditions. There are four normal types of light-emission.

The Continuous Glow. This type of luminescence, in which the light is emitted as a glow of rather uniform intensity, usually continuing throughout life, is common in lower organisms such as bacteria and fungi, and is found in the larva of *Phengodes* and in the eggs and pupae of some fireflies. As far as I know, the glow occurs normally in only one kind of mature (adult?) firefly, the larviform female of *Phengodes*. Even here, it fluctuates moderately, following mechanical stimulation.

The Intermittent Glow. In this type of luminescence, the light is emitted as a relatively steady glow which lasts for seconds or minutes. Hasama (1942c) timed the spontaneous activity of a larva of *Luciola cruciata* for 15 consecutive glows and found that the average duration was 20 seconds (range 7 to 60) and the average interval between glows was also 20 (range 4 to 86). Comparable figures were also observed for the larvae of *Pyrocoelia rufa* and *Luciola lateralis* by Hasama (1942b and c), and for *Photuris pennsylvanica* by the writer. In intermittent glowing, the light ordinarily takes several seconds to increase from zero to maximum intensity, and a comparable period to disappear. In larvae of *Photuris pennsylvanica*, however, I have seen the light appear or disappear in approximately a second. Intermittent glowing is apparently under voluntary control, although quite often there seems to be no correlation between luminescence and either activity of the animal or external conditions. Such glows can often be intensified by mechanical stimulation of the animal. Intermittent glowing is characteristic of the genera *Phrixothrix*, *Diphotus,* and probably *Lampyris,** and of the larvae of most fireflies. It also describes well the light-emission in *Pyrophorus,* although Harvey (1931) reported that, after a given glow has passed through its plateau phase and entered the decay phase, a rhythmic fluctuation, with a period of 0.8 to 2 seconds, and an amplitude variation of 25 per cent or more, can sometimes be observed "after the light has nearly subsided." Heinemann (1872) apparently observed the same phenomenon. Harvey ascribed these fluctuations to direct nervous stimulation, although he stated that they might possibly be caused by "some muscular mechanism connected with local distribution of air. . ."

The Pulsation. Hasama (1942a, 1942b) described the light of *Pyrocoelia rufa* and *Luciola lateralis* as being emitted in pulses, averaging, respectively, 6 to 13 and 60 to 110 per minute, with slight sex differences. The pulses are usually fused, thus giving the effect of a continuous light fluctuating fairly regularly. There are also occasional periods of darkness. Schultze's description of a rhythmic ebb and flow in the light of the male of *Lamprorhiza splendidula,* indicates that this species may belong here, too,† but no information accurate enough to justify including any other species is available. In regard to frequency, Gerretsen's *Luciola vitticollis* (60-120 per minute) resembles *Luciola lateralis,* as do several of the Oriental and East Indian forms mentioned in many of the extraordinary reports of synchronous flashing (Buck, 1938). However, no further data are available on their types of luminescence.

As will be seen shortly, some forms of flashing also show pulses, but, for the moment, these are considered to be basically different from "the pulsation", because (a) their frequency is very much higher, (b) they occur as part of a rigid, regularly repeated pattern, each burst being followed by several seconds of darkness, and (c) the increase and decrease of luminescence is abrupt. On the other hand, the pulsation may not prove to differ fundamentally from the intermittent glow, in view of Harvey's (1931) photocell string galvanometer studies on *Pyrophorus.* He found that the apparently steady bright plateau glow actually often fluctuates at a frequency of 2½ to 5 cycles per second, though with an amplitude variation (5%) which is too small to be detected by the human eye. For the time being, however, the category is useful for those forms which are neither typical glowers nor typical flashers.

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* It is extraordinary that so few of the early workers troubled to describe directly the type of light-emission of their material, or to differentiate between the emissions of different types of organs, such as the lateral tuberculate and the ventral organs of the female of *Lamprorhiza splendidula.* **Spallanzani,** and **Carus** (1864) mention that the larval light of "*Lampyris*" is continuous, but this is opposed by tenuous allusions here and there in the literature. For the emission type of the adult, we have only the indirect statements of **Owsjannikow** (1868) that the light of the male of *Lampyris noctiluca* "is intermittent, though some lighted for hours"; of **Wielowiejski** that "after the stopping of intense glow a weak shimmer is seen"; and of **Bongardt** that "I have never observed that the Lampyridae can suspend their light suddenly". **Bellesme** states that the female of *L. noctiluca* requires 12 seconds for control, whereas the larva can extinguish its light "suddenly" (2 to 3 seconds). This indicates that at least *Lampyris* is of the intermittently glowing type.

† **Dahlgren** writes of the female of *Lamprorhiza* as glowing continuously and the male as flashing, but it is not apparent that he observed either sex critically.

The Flash. This is the most familiar normal type of light-emission in American fireflies and is seen in many of the common lampyrids (*e.g., Photinus, Photuris, Luciola italica?*[*]). In its simplest form, the flash consists of a burst of light of much greater intensity and much shorter duration than occurs in a glow. Ordinary observations show that the light intensity rises abruptly from zero to a maximum and then declines abruptly again to zero, but the duration of the flash is ordinarily so short that no accurate idea of the phenomenon can be obtained with the naked eye.[†] Using sensitive photocells in combination with amplifiers and a string galvanometer or oscillograph, Brown and King (1931), Snell (1932) and Alexander have carefully studied the "normal" (*i.e.,* laboratory) flashing of *Photinus pyralis* and *Photuris pennsylvanica.* These studies show, among other things, that the duration of the flash is of the order of 0.1 to 0.2 seconds and rather constant, intraspecifically; that the peak intensity and total amount of light emitted per flash are quite variable; and that the augmentation and decay phases are fairly symmetrical (FIGURE 41). The extensive photometric work of Coblentz established 1/400 candle as the light intensity of an average flash of *Photinus pyralis.*

The limitation of most recent experimental work to *Photinus pyralis,* which has a simple single flash, and *Photuris pennsylvanica,* which seems to have a single flash under laboratory conditions, tends to obscure the fact that many much more complex types of flash exist. McDermott (1917) summarized the characteristic flash types given during normal flight by nine American species. Most of these are single flashes of various durations and relative intensities. However, the male of *Pyractomena lucifera* and both sexes of one variety of *Photuris pennsylvanica* emit flashes with multiple peaks, instead of the simple "normal curve" type of luminescence (FIGURE 41). These flashes (better, "coruscations" or "twinkles") can be construed either as due to an optical fusion of separate flashes occurring close together or as rapid fluctuations in peak intensity of a single flash. I have records similar to McDermott's of the flashing characteristics of about forty species of Jamaican fireflies, of which eight show a coruscating type of flash. These multiple flashes differ interspecifically in duration, and in the frequency of the individual peaks, and in some species occur in complex, but constant, combinations with single flashes. The maximum frequency of the oscillations in intensity during a coruscation is not known, but is certainly not much less than the critical flicker fusion frequency of the human retina (probably from 25-30 cycles per second under the conditions of observation). Lund's description of

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[*] In *Luciola italica,* **Emery** described the "increase and decrease of the light in short regular intervals," and **Verworn** (1892) described the "rhythmic intermittency" of the light of both sexes in flight. The frequency was 60 to 80 per minute according to **Verworn,** and 80 to 100 per minute according to **Peters** (1841). **Verworn** mentioned a weak continuous glow, given while resting, and stated also that the light was not completely extinguished between flashes. This description fits pulsing as well as flashing, but **Geipel's** "strict rhythm of flashing and extinction" indicates that *L. italica* is a flashing type.

[†] Occasional individuals of flashing species show a very faint glow which persists between flashes.

"regular, rapid and numerous changes in intensity" in the flashing of two Jamaican species of *Photinus* almost certainly refers to such coruscations.

It is possible that even the apparently single flashes of some species may show finer cyclic oscillations similar to those discovered in *Pyrophorus* by Harvey (1931). In *Photinus xanthophotis catherinae,* which has a very large organ and a flash lasting about half a second, I occasionally saw, with peripheral vision, very slight and very rapid fluctuations. However, it is also possible that a subjective effect is involved in this, since it is well known that, with light of very low brightness, the eye tends to shift its retinal fixation point rapidly. Such an effect might also be the explanation of the *"ébranlement particulier"* of the "retina" which Bellesme reported in close observation of the light of *Lampyris* in a darkroom.

In many flashing types of fireflies, light-emission is used in astonishing systems of mating signals in which the male flies about, flashing at regular intervals, while the female, usually at rest, flashes in response to his signals (Osten-Sacken, 1861; McDermott, 1910, 1911a, 1912, 1917; Mast, 1912; Buck, 1937b). These signal systems are remarkably precise in their time relations and differ characteristically in different species. In normal flight, the male produces his luminous unit (whether single flash, coruscation, or complex) at regular intervals, and this emission pattern is so characteristic that the males of each of the dozen or more species which may be active at night in the field at the same time can be identified reliably from observation of their flashing, alone. As might be expected, the frequency of flashing rises with temperature (Snyder and Snyder, 1920; Buck, 1937b).

In summary, these four types of light-emission can be interpreted as the results of four progressively more effective modes of control. The continuous glow is an indication of the inability of the animal to prevent the luminescent reaction, or even to change its rate materially. In the intermittent glow type, the organism can interrupt photogeny, but only slowly, and the plateau level of glow presumably represents the condition with no control operating. In the pulsation, a further refinement of control may be assumed to have developed, so that now the light can be made to fluctuate fairly rapidly. In the flash, finally, the control mechanism achieves its highest development, as indicated by its ability to bring about bursts of light of very quick accretion and decay, of short duration, and with complete extinction between even closely spaced flashes. Since, in most flashes, nothing resembling a "plateau" is reached, it is uncertain whether or not the peak of the flash represents maximum possible luminescence.

With the above hypotheses in mind, it is interesting to review these various types of light-emission in connection with the morphological studies already discussed. In the continuously glowing *Phengodes,* the luminous cells lack any specialized tracheal supply and presumably obtain oxygen from the body fluid with which they are bathed. Tracheae are also absent in eggs, and presumably also in pupae, because of histolysis. In the organisms with intermittent glows, we find, exclusively, organs of Types 2 and 3, with a branched and tapering tracheal supply lacking end-cells. In the fireflies with the pulsating type of light-production, we find end-cells present, but with arborescent tracheal branching, as described for organs of Type 5. Finally, in the flashing type, we find the cylinder, the highest morphological development in the sense of precision and complexity of organization. Thus, there seems to be a striking correlation between the type of tracheal supply in the photogenic organ and the normal type of luminescence. In particular, the presence of end-cells seems to be associated with the ability to produce a flash or pulse. This has been pointed out previously by various workers on the Type 6 organ* and will be discussed further in connection with end-cell physiology. Whether or not the proposed distinction between the pulsation and the "true" flash will prove valid must await careful observation on further species having the Type 5 tracheal arrangement. At present, I am not able to suggest any very convincing anatomical reason why the Type 5 organ should be less abie than the Type 6 to control luminescence abruptly. Control might be connected with the cylinder itself, but it might also be related to some other anatomical feature such as, for example, the differentiated zone of the photogenic cytoplasm, or tracheolar anastomosis, neither of which seems to have been recognized in firefly photogenic organs of Type 5.

Localization of Luminescence. Microscopic observation of the active photogenic organ, preferably in the living animal, provides the only acceptable evidence of where light-production is localized. A number of investigators have reported work of this sort. Except for Weitlaner (1909), no one, since Kölliker first demonstrated the two-layered structure of the organ, has doubted that the light is produced in the "photogenic" layer, although certain workers (*e.g.*, Wielowiejski) thought that the reflector layer, too, was slightly luminescent. Almost certainly, this idea was fostered by contamination of the reflector layer with photogenic material or by diffusion of light from the underlying photogenic layer.

Spallanzani was probably the first to observe the surface of a glowing light organ. He reported having seen many tiny points of light. Kölliker and Schultze observed the same "minute sparks" in *Lamprorhiza,* and Schultze, in addition, believed that the points corresponded to his newly demonstrated end-cells. Schultze is generally credited with the claim that the light is confined to the end-cells, but actually he referred to the light as "beginning" in the end-cells. Since he also suspected a connection between nerves and "parenchyma" (photogenic) cells, Wielowiejski and Bongardt have argued that this implies his believing the photogenic cells to luminesce in addition. Bongardt and Lund described just such a spread of light from minute flashing points into a general glowing of the whole organ, and stated that the positions of the individual spots were constant, though their intensities were not. Bongardt also claimed that the number of end-cells, as determined from the points of reduction of osmic acid, far exceeds the number of lighting points, indicating that the end-cells are not the luminous points. This argument is not necessarily relevant, since not all the end-cells would need to be active at once.

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* This is apparently what Hasama (1942c) was referring to in his statement that "many authors have ascribed the strongly rhythmic blinking of tropical fireflies to the concentric arrangement of the end-cells." (Translation.)

The localization of luminescence has been much more extensively studied in the Type 6 organ than in *Lamprorhiza* and *Lampyris*. Emery set the general pattern for all later workers when he described the surface of a glowing (not sparkling) *Luciola italica* organ as showing a pattern of luminous rings, the dark centers corresponding to the cylinders. The same sort of structure has been observed in *Photinus marginellus* (Townsend), *Photuris pennsylvanica* and three Jamaican Photini (Lund), and in *Photinus pyralis* and *Photuris pennsylvanica* (Alexander). Emery discovered, in addition, that the luminous rings were not uniform but showed spots which lit up and went out irregularly and were, he thought, constant in position. Emery is a little vague about the exact localization of these spots, but it appears that he believed the light to occur at the contact between the end-cells and the photogenic cells. Lund confirmed Emery's observations in all respects and added the finding that the end-cells did not luminesce. He also discovered that when an organ was glowing brightly enough so that the individual rings fused and made the whole intercylinder area luminescent, the brightest region was in the differential zone at the periphery of the photogenic cells. Alexander, in turn, confirmed Lund's report on the localization of the bright points of light at the bifurcations of the tracheoles, and on the fact that they fire repetitively and asynchronously. This rapid, irregular, scintillating or "spinthariscope" type of local luminescence has also been observed by Wood (1939) in a firefly poisoned by a spider,* and by Kastle and McDermott (1910) and Alexander following a variety of treatments, of which strychnine injection was most effective. In addition, I have seen it following distilled water injections and cyanide vapor. Besides the glowing rings and sparkling points, "phosphorescent clouds" which sweep across the organ in waves have been described (Emery, Kastle and McDermott, Lund). According to Lund, they originate deep in the tissue.

The observations just described were used by Lund to support his claim concerning the enzymatic activity of the periphery of the tracheole, which has already been mentioned, and by Emery to further his idea that the photogenic material "secreted" in the light cells is transported to and burned at or in the end-cells. It should be kept firmly in mind, however, that no details of any kind can be made out in an organ which is flashing, or even exhibiting a bright glow, and that the conditions under which the luminous rings, scintillations, and cloudy waves are seen are distinctly abnormal. Moreover, it must be remembered that none of the findings have any necessary relevance for organs of Types 2 and 3, where end-cells and cylinders are lacking.

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* Steche (1908) observed normal intermittent flashing in a firefly apparently paralyzed by spider bite.

In contrast to light organs which contain end-cells, the organs of Types 2 and 3 show only a uniform and structureless glow. Dahlgren and others have pointed this out as characteristic of *Pyrophorus,* "the larvae of all Lampyrids", "the females of *Lamprorhiza splendidula* and all species of *Phengodes*". From personal observation, I can confirm Dahlgren's statement on *Pyrophorus* and the larva of *Photuris pennsylvanica.* I would expect the statement about *Lamprorhiza splendidula* to apply only to the lateral tuberculate organs. In *Phengodes,* I have described the light as coming from minute separate spots corresponding in number, size, and position to the huge oenocyte-like cells seen in histological preparations (FIGURE 15; Buck, 1946a).

The theories of Schultze and of Emery, which gave the end-cell a primary role in photogeny, were based on two observations: the presence of points of light in the general region of the end-cells in the living light organ, and the affinity of the end-cells for osmic acid vapor. They also involved the assumption that a high reducing power for osmium indicates a high affinity for oxygen. We have already seen that Lund denied that the end-cells actually luminesce, and that several workers have described the spread of luminescence throughout the photogenic cytoplasm. Nevertheless, observations on the living organ are not precise enough to rule out the possibility that the end-cells, or other inter-cylinder material, may light in addition to the photogenic cells. This is particularly relevant since so many reports speak of the luminescence beginning at the edge of the cylinder and since Lund himself admitted that the end-cells are often situated in little bays hollowed into the photogenic cytoplasm. The point about the site of reduction of osmium, which Emery regards as the *"experimentum crucis,"* and which Lund also uses to bolster up his theory of the enzymatic activity of the tracheolar wall, has been dealt with on page 409. The particularly heavy deposit does not necessarily prove that there is anything unique about the end-cell, but merely that it stands at the first spot permeable enough to allow the vapor to escape. The slight penetration into the tracheoles would then be caused by the osmium being reduced as fast as it arrives at their proximal ends. Wielowiejski has argued, in addition, that a high affinity for oxygen does not necessarily identify the site of photogeny, and has made the interesting suggestion that the end-cell acts in a manner analogous to the red blood cell and "stores" oxygen. Wielowiejski's and Emery's theories thus each provide that material, freed in the photogenic cells by nerve stimulation (the control), moves toward the end-cell, by a method not elaborated, and luminesces near it because the highest concentration of oxygen is found there. However, no evidence exists to show that any oxygen carrier is present in or around the end-cell, and in any event its activity would be an effect, rather than a cause, of luminescence.

Tracheolar Properties of Interest in Regard to Control of Luminescence. Creighton injected adrenalin into *Photuris pennsylvanica* and observed a bright glow. This, as we have seen, is the usual response to dozens of agents. On the basis of histological studies, he claimed that the adrenalin effect was not hormonal but was due to contraction of muscle fibers in the tracheal end-cell which enlarged the part of the tracheole within the end-cell, as postulated by Dahlgren. However, according to a personal communication cited by Alexander, it appears that Creighton's preparations showed a dilation not of the end-cell lumen but of the whole tracheole. We have, then, the possibility that luminescence may be controlled by an active or passive dilation and constriction of the tracheole, such as Gerretsen suggested as an alternative to end-cell activity.

Many have regarded the fact that the tracheoles do not dissolve in caustic solutions as proof that they are composed of chitin, although this has not been confirmed by more specific tests. If the walls of the tracheoles were composed purely of chitin, they would be unlikely to be very dilatable. However, as Richards (1947) has pointed out, protein often bulks large in cuticle and could satisfy most conditions for extensibility. It would be desirable to have better evidence of the chemical composition of the tracheoles, but color tests would not be conclusive, in my opinion, because of the difficulty in getting a strong enough color in a structure as thin as the tracheolar membrane. For many years, the absence of visible spiral thickenings in the walls of the tracheoles was regarded as strong evidence that they differ from the ordinary larger tracheae. However, Richards and Anderson (1942) have shown by electron microscopy that honeybee tracheoles have spiral thickenings at least down to a diameter of 0.2 micron. The presence of thickenings in a tracheole might reduce the extensibility of the wall, but would not prevent tracheol collapse, as Richards and Anderson have shown.* However, if Creighton's finding of tracheoles dilated by adrenalin is accepted, it indicates that the chitin content is low.

There is an opinion that tracheoles must differ in composition from tracheae because they are permeable to gases and liquids. This idea probably stems from Krogh's (1919) evidence that the diffusion rate of oxygen through chitin is only about one-thirtieth of that through water, and about one-tenth of that through tissue. However, if it is recalled that the membrane of the tracheole is only between 0.005 and 0.01 micron thick (Richards and Anderson), it will be seen that simple transfer could go on readily even through chitin. Wigglesworth (1930) and others have, in fact, shown that tracheae are freely permeable to oxygen and nitrogen.

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* References to additional literature on tracheoles will be found in **Wigglesworth** (1931 and 1939), and **Richards & Anderson.**

Hoskins (1940) has called attention to one possibly valid distinction between tracheae and tracheoles, which is that the former are as a rule distinctly hydrophobic, whereas the latter are often strongly hydrophilic. Even here, some confusion exists, since it has been reported that the tracheae are hydrophilic in newly moulted insects (Wigglesworth, 1938b) and, conversely, that oil which enters the tracheae eventually penetrates into the tracheoles (Hoskins).

It is almost universally held, either explicitly or implicitly, that the principal function of the tracheoles in the light organ is to conduct oxygen to the photogenic cells. It should be emphasized that, however reasonable this view may appear, it is only an assumption. Moreover, there has not even been a serious attempt to define, theoretically, the conditions under which oxygen conduction might take place. Among the more obvious items of needed information are data on whether transport occurs by diffusion or by flow, and whether the tracheoles contain air or liquid. A further discussion of theoretical aspects of these questions will be found in the section on end-cell physiology. For the present, we can conclude only that, if transport is by diffusion, the tracheole loses most of its meaning if it is assumed to be filled with liquid, because the diffusion of oxygen down a column of water is no faster than it would be in free water, and only about three times as fast as through dense connective tissue (Krogh). Only by containing air, in which oxygen diffuses thousands of times as fast as through water, could a tube confer any advantage in supplying oxygen. To be sure, if the tracheole were absolutely impermeable to oxygen for some of its length, access of oxygen to parts of the photogenic cytoplasm could be prevented or delayed even if the tracheole were liquid-filled, but it is hard to see what advantage would be conferred thereby and, moreover, we have already seen that impermeability to oxygen is extremely unlikely.

Another possibly significant point is Wigglesworth's (1930) observation that air bubbles, trapped between two columns of oil advancing toward each other in a trachea, are completely squeezed into solution, in the hemolymph or tissue surrounding the trachea, by the surface tension of the oil. I have seen the same thing happen in dipteran larvae immersed in kerosene. An astonishing feature of the process is its speed. The factors involved are too complex to permit one to say off-hand whether the same phenomenon would occur in tubules of the dimensions and structure of tracheoles, or, if so, whether it would be rapid enough to meet the requirements of photogenic control. However, the principle should be kept in mind as a possible method of forcing oxygen into cells. If, for example, the tracheal end-cells were to "close", and if water moved up the tracheole proximally, it might drive some of the contained air into the photogenic tissue. This would be the reverse of the theory of osmotic control of luminescence to be discussed in a later section.

The Nervous System and Luminescence. A connection between the nervous system and luminescence has long been accepted by most investigators, on the basis of the anatomical evidence already discussed and because of a variety of experimental work. It was shown very early that decapitation, or cord section anterior to the organ, results in immediate cessation of voluntary flashing or glowing, though a dim constant luminescence may persist, or reappear some time later (Macaire, 1821; Peters, 1841; Verworn, 1892; Dubois; Prowazek, 1908; Lund, Williams). Likewise, luminescence is initiated or increased by stimulating the nerve cord[*] mechanically (*e.g.*, Heller, 1853; Verworn), or electrically (Macartney, 1810; Macaire; Todd, 1826; Joseph, 1854; Kölliker; Owsjannikow, 1868; Bellesme; Heinemann, 1886; Dubois; Fuchs, 1891; Steinach, 1908; Lund, Gerretsen, Perkins, 1931; Snell, 1932; Brown and King, Alexander), even after section proximal to the point of stimulation.[†] Direct electrical stimulation of the photogenic tissue likewise causes lighting. Nervous influence is also suggested by numerous observations that anesthesia suppresses voluntary control of luminescence,[‡] and by the apparently stimulating effects of spider venom (Wood), various neurotoxins (Kuhnt), and DDT (page 75). In all these instances, however, direct action of the agent on the photogenic tissue, or on the tracheal system, cannot be excluded.

In contrast to the above work, Owsjannikow observed no diminution of light in isolated organs after 1½ hours' soaking in curare and strychnine, and concluded that the nervous system is not involved in luminescence. Bongardt repeated this experiment, using the entire animal, and reported no effect in 12 hours. Aside from the questions of penetration and of whether vertebrate poisons would be expected to act on insects (raised also by Bongardt), this conclusion involves a type of *non-sequitur* encountered frequently in the older work, and well illustrated also by Bongardt's argument that fireflies cannot stop their light suddenly, because dead ones continue to glow for 10 to 20 days. Owsjannikow and Bongardt thus failed to see that what happens in the injured or dead animal may be entirely irrelevant to the question of whether the nervous system, or anything else, permits voluntary control of luminescence in the normal living animal.

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[*] Because of the minute dimensions involved, it is extremely likely that simultaneous stimulation of other tissues near the operated region was not excluded, particularly in the earlier work.

[†] Some of these papers report considerably more than the bare observation. They are interesting from the standpoint of comparative electrophysiology.

[‡] However, luminescence itself may persist for long periods under conditions where voluntary neuro-muscular activity is suppressed or abolished entirely.

Harvey's (1931) photocell-string galvanometer records on the glowing of *Pyrophorus,* which have already been discussed, indicate that the light is under nervous control, particularly as Harvey was careful to exclude respiratory and pulse movements. A better test could be made by recording simultaneously the action potential pattern of the ventral nerve cord, and the luminescence, particularly if done on several species with differing flash patterns. It may be of interest here to mention some other potentials associated with luminescence, though not necessarily with the nervous system. Many years ago, Kölliker obtained some "not quite constant" evidence that lighting fireflies deflect a "multiplier" (galvanometer) more than do non-luminescent ones. In view of Kölliker's primitive apparatus, his report does not call for extensive consideration. Recently, however, Hasama has reported that, in a number of Korean species, the light organ itself produces a monophasic action potential during activity. The luminous segments are electronegative to the non-luminous. In the larva, the potential pattern is continuous, and synchronous with light emission. In the adult, it exhibits rhythmic or cyclic fluctations, the frequency of which corresponds satisfactorily with that of the pulsing of the light in *Pyrocoelia rufa* (13 per minute), but poorly in *Luciola lateralis* (48 per minute for the potential, 60 per minute minimum for the light). With the electrodes on two non-luminous segments, instead of on one luminous and one non-luminous, no potential difference was detected, thus indicating that muscle potentials, etc., are not involved.

A number of further lines of evidence indicate indirectly that the nervous system is concerned with the control of luminescence. For example, the rigid characteristic species flash-patterns already referred to, as well as the inherent diurnal rhythm of luminescent activity* (Allard, 1931; Perkins; Rau, 1932; Buck, 1937a), point to the existence of at least an involuntary center of nervous control. Indeed, Verworn, some sixty years ago, postulated in detail that normal luminescence is under absolute control of an automatic nervous center in one of the two most anterior ganglia of the cord. Though similar hypotheses have since been used by several writers in attempting to explain synchronous flashing (see Buck, 1938), work on the mating signals shows that a high degree of voluntary control can also be exercised. Briefly, these signal systems enable the female of one species, or an artificial light operated in a specific way, to attract males of the same species without attracting males of another species. Conversely, males of a given species signal only to a female of the same species (or to a properly operated artificial light) and ignore other males of their own species, both males and females of other species, and all improperly executed artificial signals. In *Photinus pyralis,*

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* This periodicity may play an important and unsuspected part in certain experiments, since **Maloeuf** (1938) and **Alexander** have claimed that it influences reactions as basic as the appearance of luminescence in high partial pressures of oxygen.

I have shown that the ability of the male to recognize the female depends on the fact that she always replies to his flash after a particular time interval (Buck, 1937b).

The fact of nervous influence, however, does not solve the problem of the control of luminescence, since the effect could either be direct (*i.e.,* stimulation of the photogenic cells), or indirect (*e.g.,* stimulation of the end-cells; oxygen regulation). On this question there is no dearth of opinions, pro and con, but valid empirical evidence has proved to be extraordinarily hard to obtain.

Evidence that oxygen regulation is the primary factor in control of luminescence is, of course, opposed to the idea of direct nervous stimulation of the photogenic cells. This evidence will be analyzed later in detail (page 441), but it may be said here that it is insufficient to exclude the possibility of direct nervous control.

Heinemann (1886) reported that electrical stimulation of nerves anterior to the abdominal photogenic organ of *Pyrophorus,* after all other tissues of the trunk had been transected, did not induce luminescence. From this he concluded that the nerves act by way of some intermediate tissue. However, since it was a negative type of experiment, and since Lund, in repeating it, obtained precisely the opposite result, it cannot be regarded as decisive. It is also difficult to reconcile with numerous reports of luminescence obtained by electrical stimulation of isolated abdomens of lampyrids, and with the experiments of Fuchs on localized stimulation of the photogenic organs and nerves of *Pyrophorus.*

Considerable evidence has been adduced in favor of direct nerve action. Lund's transection experiment has already been mentioned. However, Lund's acceptance, on the basis of this and other work, of "primary control of the organ . . . by nerves in direct connection with the photogenic tissue" is greatly weakened by a number of apparent contradictions in his presentation. For one thing, it appears that, in spite of denying oxygen a main role in control, he actually considered the end-cell to be of prime importance. For another, he emphasized the well-known fact that the photogenic tissue itself "is irritable and responds locally to mechanical stimuli", overlooking the probability that such disturbances also facilitate access of oxygen. Steinach, using induction shocks applied directly to the photogenic organs of decapitated specimens of *Lampyris,* claimed to have demonstrated summation of subliminal stimuli for luminescence. He attributed the summation to a direct effect of the nerves on synthesis of substrate for luminescence, and rejected the alternative idea of simple neural summation, although his reasons for so doing are unconvincing. Bellesme also supported direct nervous stimulation of synthesis on the basis of an experiment in which he found that fireflies cut open in air luminesced sooner, if they had first been "stimulated" electrically in the absence of oxygen, than if they had not. However, aside from the unavoidable variations in manipulation, the temporal variability in responses of fireflies under such conditions is ordinarily so large that a conclusion cannot be accepted until verified on a considerable number of specimens. Moreover, no allowance was made for the probable effects of the anoxia upon the nerves. Additional details concerning the work of Steinach and Bellesme will be found on pages 438 and 441. Work of Snell bearing on the same problem is considered on pages 442 and 443.

Perhaps the strongest evidence of direct nervous control of luminescence is furnished by the numerous experiments on electrical stimulation of *Pyrophorus* and of firefly larvae. Here, the finding of increased intensity of luminescence with increased intensity of stimulus is less easy to attribute to oxygen control, since these forms lack end-cells.

The problem of direct *versus* indirect nervous control of luminescence is thus unsolved, although there is much evidence in favor of the former alternative. Unfortunately, the effects of oxygen and nerve action seem inseparable, experimentally. Under abnormal laboratory conditions, it is clear that nerves are not essential for luminescence, since fireflies will glow under a variety of conditions incompatible with nerve action, or even with life, if oxygen is present. Nevertheless, as we shall see, there is no conclusive evidence that oxygen is ever limiting in normal flashing, and there are many indications that control is much more circuitous than by direct regulation of oxygen access to the photogenic tissue. A number of hypothetical mechanisms of intracellular control, such as might be set off by direct nervous stimulation, are discussed on pages 434 to 436. Moreover, it should be kept in mind that the fact that the experiments discussed are inconclusive, does not disprove direct nerve action. The interpretation of Bellesme and Steinach may yet turn out to be correct, even though it does not necessarily follow from their experiments.

THE CONTROL OF LUMINESCENCE

Historical. Before entering on the detailed discussion of modern work and theories on control of luminescence, it may be interesting to consider, briefly, a few of the older theories proposed during the long history of the study of fireflies, and now abandoned. Perhaps the oldest and most persistent idea was that luminescence is controlled in some way by "respiration", by which apparently was meant that the muscular respiratory movements pumped air into the photogenic organ (Joseph, 1854; Faraday, 1814; Carrara, 1836; Siebold, 1848; Leydig, 1857; Seaman; Heinemann, 1886; Watasé). Several of these observers maintained that the breathing movements are synchronous with light-emission. However, breathing movements could hardly be the only control, unless the light pulsated day and night or the insect stopped breathing by day, neither of which alternatives seems likely. Elaborations on the idea were proposed by Carrara, who claimed to have found an air tube leading from the mouth to an abdominal bellows or vesicle; by Heinemann (1886), who envisaged the abdominal musculature as pumping air through the "hiatuses" in the photogenic layer of *Pyrophorus;* and by Seaman, who suggested that the spiracles were first closed and air was then forced into the organ by abdominal contraction. A serious stumbling block for these theories is the fact that most modern workers have been unable to observe any visible muscular movements coinciding with luminescence (Lund; Harvey, 1931; Hasama, 1942a). Likewise, Heinemann's theory has little support, since Geipel has shown that the musculature of *Pyrophorus* is in no way different from that of non-luminous elaterids, and since, as previously stated, the existence of the spaces in the photogenic layer is very dubious. The same objections apply to Dubois' theory, according to which not air but blood was supposed to be percolated through the photogenic organ of *Pyrophorus.* The circulatory system was also implicated by Carus, who maintained that the rhythm of luminescence corresponded to the pulse, and by Tozzetti, who suggested that the tracheoles carried part of the blood.

Another theory was to the effect that the light-emission was controlled by withdrawing the light organ into the interior of the abdomen, where it was concealed by other viscera (Carradori, 1797; Müller, 1805; Owsjannikow, 1864).

Deductions from Kinetics Analysis. By high-speed recording, Brown and King, Snell and Alexander obtained time-intensity curves of the flashing of *Photuris pennsylvanica* and *Photinus pyralis.* Snell pointed out that the form of these curves is determined by three variables: the number of photogenic units active, the degree of activity of each unit, and the degree to which each unit is stimulated.

If these curves represented the response of a single photogenic unit, or the simultaneous responses of all the photogenic units in the light-organ, their analysis could reveal valuable information on the luminescent reaction and its control mechanism. From the form of the accretion and decay phases, it might be possible to deduce, for example, the number of reactants involved in the rate-determining reaction and the number of units active; and to ascertain whether diffusion or "phase-boundary removal" (see page 436) brings the reactants together; whether or not the unit response is of the all-or-none type; whether the control mechanism "opens" by relaxation or contraction; etc. Brown and King, in fact, suggested a relation between the supposed logarithmic form of the decay phase of the flashing curve, and the similarly logarithmic form of the decay phase of the luciferin-luciferase reaction (*Cypridina*) *in vitro.* According to Snell, however, a logarithmic form is the exception rather than the rule. Snell found the durations of normal flashes very constant, the intensities highly variable, and even the reaction velocities (slope of decay phase) different.

The two segments of the luminous organ of the male of *Photinus pyralis* contain about 6,000 cylinders, each of which encloses a minimum of 80 to 100 end-cells, which, in turn, give off twice that many tracheoles. The tracheoles supply an estimated total of 15,000 photogenic cells, each of which is in contact with two cylinders (FIGURES 13 and 31). In studying the glows produced by various agents, I have noticed many times that single cylinders may light as individual units—that is to say, the surface of the organ may show minute isolated "doughnuts" of light which glow on and off independently. We must allow, then, for at least 6,000 photogenic units. If we accept Lund's and Alexander's claims that the end-cell (region) can flash independently of the photogenic cells, as in "scintillation," the number must be increased to something like 600,000. In either case, there is a strong likelihood that the time-intensity curve for the flash of the whole organ represents not the result of an absolutely synchronous firing of all the units, but the statistical result of the firing of units slightly out of phase. This is supported by the suggestive resemblance of the time-intensity curve to the normal-distribution curve. If the curve is indeed a statistical one, we can draw the very important conclusion that the duration of the luminescence produced by a single photogenic unit must be far less than the 0.15 second average for the mass flash. We have no way of knowing what the duration of the flash of the individual luminescent unit (as distinguished from its glow) may be, but it can hardly be more than one-tenth of that of the collective flash. The importance of this inference is that it imposes much more severe conditions upon the control mechanism. We now require a mechanism capable of producing isolated flashes of light with durations of the order of 0.01 second, or less. The same conclusion is indicated by the observations previously reported on the repetitive or oscillating types of flash seen in some Jamaican fireflies and in *Photuris pennsylvanica* in the field, although, here, there remains the possibility that the coruscations could be produced by groups of units firing successively in relays.

Mechanisms of Intracellular Control. We have seen that there is no agreement on whether flashing is controlled primarily by the nervous system or by the oxygen supply system. There is also no agreement on whether these two systems operate directly, *i.e.*, on the photogenic cell, or indirectly, *i.e.*, on each other. It is therefore appropriate to consider what general types of mechanism within the photogenic cell might be available as a basis for assumed "direct" effects of nervous or oxygen stimulation.

The protozoan *Noctiluca* is described (Quatrefages, 1850; review in Harvey, 1940) as emitting a bright flash, consisting of the momentary lighting of a multitude of tiny sparks scattered through the protoplasm, each representing a granule of photogenic material. The flash is not repetitive or voluntary, in the sense of being initiated internally, but is a direct response to some environmental stimulus (osmotic, thermal, chemical, mechanical, etc.). The synchronous lighting of the cytoplasmic granules during the flash is presumably attributable to the fact that the stimulus reaches the cell through the ambient water and, hence, almost simultaneously at all points on the surface. The bare essentials for controlled luminescence thus seem to be, first, the presence of a chemiluminescent system in the cytoplasm; second, a source of the necessary raw materials (here obtained from the surrounding sea water); and, third, a stimulus which will set off the intracellular reaction.

Harvey (1940) has pointed to the fact that a single cell like *Noctiluca* can flash as showing that complicated physiological mechanisms, such as those in the firefly, are not necessary for the control of luminescence. He thus regards the response of *Noctiluca* as quite similar to the responses of other cells (*e.g.*, muscle) to direct stimulation, and implies that the response of an individual photogenic cell of the firefly need not, intrinsically, be any more complex. According to Harvey's view, therefore, the nervous system would correspond to the environmental changes which stimulate *Noctiluca,* and, in the firefly, would represent the anatomical and physiological answer to the problem of controlling the responses of thousands of *Noctiluca*-like photogenic units. The tracheal system, likewise, would stand in the same relation to the photogenic tissue as the sea-water to *Noctiluca,* and would be an arrangement for attaining, throughout a massive tissue deep within a body, conditions of aqueous diffusion of oxygen comparable to those of a free cell suspended in aerated sea-water. We may, therefore, think of *Noctiluca* and the photogenic cell of the firefly as presumably equivalent in regard to their intracellular control mechanisms.

Before considering possible intracellular controls, however, it is well to emphasize that the nervous and tracheal systems of the firefly, subsidiary though they may be, have important influences on luminescence. They are, therefore, as much a part of the control problem as the intracellular activities, and can be investigated independently of the latter.

A number of intracellular control mechanisms have been proposed, all on largely speculative bases. Perhaps the simplest of these, which may be called the "reactant limitation hypothesis", postulates that luminescence is controlled by limiting one of the four fundamental components of the luminescent reaction. With respect to oxygen, this hypothesis is equivalent to direct external regulation, but in regard to the other three essential reactants, even our very limited knowledge of the chemistry and enzymology of luminescence is sufficient to suggest various intracellular processes which might limit light-emission. Various reports indicating control by substrate synthesis, or storage and release, are considered on pages 437 to 440.

A second intracellular control mechanism has been proposed by Harvey on several occasions, and may be called the phase-boundary hypothesis. This hypothesis provides that all the essential reactants are present in the cell at the same time, but are prevented from reacting with each other by phase-boundaries. When these boundaries break down, as might occur as a result of nervous stimulation of the cell, the reactants can come together and produce light. Phase-boundary phenomena are well known in physical chemistry, and there are a few suggestive physiological and biochemical analogues, but we are still far from having a clear idea of how potential reactants are segregated from each other in the living cell. In fact, phase-boundary changes may only be some subtle form of reactant limitation. A single test of the phase-boundary hypothesis has been made on fireflies, but, as described on pages 442 and 443, the results were not conclusive. The similarly inconclusive experiments of Bellesme and Steinach on direct nerve action (pages 432 and 437) can also be interpreted as consistent with phase-boundary control. For the present, however, the concept must be considered simply as a theory, albeit a reasonable and stimulating one.

A third hypothetical intracellular control mechanism is one which makes use of a possible competition for oxygen between respiration and luminescence. This hypothesis, which has a number of variants, and which has not been tested experimentally, will be discussed on page 448.

None of the above discussion of intracellular mechanisms bears on the validity of the commonly held view that nervous control is direct and the tracheal system merely a means for ensuring that adequate oxygen is always available for luminescence. We shall be able to judge this better after having considered the evidence on oxygen limitation.

In conclusion, on *a priori* grounds it appears that intracellular control mechanisms would have a considerable advantage over external control mechanisms (*e.g.,* tracheoles or end-cells) in regard to speed of action. This might be crucial in the control of high-frequency light-emissions, such as are seen in the coruscating types of flashing. On the other hand, by making the control of luminous intensity more or less independent of changes in external oxygen tension, doubt is cast on the generally accepted significance of certain anatomical features of the photogenic organ, particularly the end-cell.

Water as a Possible Limiting Factor in the Control of Luminescence. Some of the earliest experiments demonstrated that if a firefly or an extirpated organ is quickly and thoroughly dried, it can be preserved in a non-luminescent state for long periods and will glow again when moistened (Spallanzani, Carradori, Macaire, Kölliker, Carus; Owsjannikow, 1868; Dubois, Bongardt, Kastle and McDermott; Hasama, 1942a). However, it seems extremely unlikely that water lack is ever a direct factor in controlling light-emission in the living animal, since this would appear to demand a high-frequency cyclic dehydration and rehydration of the photogenic cells, and since luminescence continues for a considerable time in minced organs, where dehydration must be severe. This, then, is a good example of a method of limiting the luminescent reaction *in vitro* (or at least in the absence of living protoplasm) which is not involved in the normal control of photogeny.

Luciferin and Luciferase as Possible Limiting Factors in the Control of Luminescence. In discussing various theories of photogenic control by limitation of reactants, it should be understood that the terms, *photogenic material,* or *substrate,* could refer either to luciferin or luciferase, since there is no evidence which points specifically to either.

If either luciferin or luciferase were not in the luminous organ, the problem of control could be simplified. It was, in fact, a convenient feature of Dubois' control theory that the luciferase was brought to the organ by the blood which circulated through the interstices of the photogenic layer and there met the luciferin. In all modern speculations on luminescence control, however, it has tacitly been assumed that these two reactants are formed in the photogenic layer and remain there, and that control is therefore a cellular problem. Moreover, most writers have assumed that photogenic materials are usually stored in the photogenic organ in excess, and that synthesis of substrate has no role in rapid control of luminescence. This view is based on the aforementioned observations that dead, minced, or even dried and remoistened fireflies or organs will glow for many hours. Since, however, the enzyme luciferase seems to survive even drying, there is no *a priori* reason why synthetic or other enzymatic methods of releasing substrate might not also survive.

In considering luciferin or luciferase as a possible limiting factor in luminescence, it is necessary to distinguish clearly, as Bellesme and Gerretsen have done, between the reactions which form the luminous material and those in which luminescence takes place. This distinction has often been overlooked. It is also necessary to differentiate carefully between mechanisms which operate to keep luciferase or luciferin separated (both being present) and those which limit the rate of formation of one or the other. The phase-boundary control mechanism, based on the former hypothesis, has already been discussed (p. 432). The latter hypothesis, according to which the rate of synthesis of "photogenic material" in the photogenic cells may become limiting, has also been debated, though not always in a clearly defined form. Steinach made implicit use of the hypothesis when he reported that stronger electrical stimulation produces a stronger light because more "light-stuff" is formed. Bellesme supported the same hypothesis explicitly when he postulated that the photogenic cells secrete a gaseous product (phosphine!) which lights as soon as it comes in contact with air. On the basis of the rather dubious claim that a thoroughly crushed glowworm does not glow (when it ought to, since its interior is exposed to air), Bellesme postulated, in addition, that no lighting material is stored in reserve. In further support of this, he reported that there was a latent period between stimulus of the animal and the beginning of luminescence, and that this decreased if the firefly was first stimulated electrically in the absence of oxygen, so as to build up a store of luminous material. Delay might very well be seen in glowing forms, where the build-up of luminescence is slow, but, as we shall see, it is more likely to have another explanation. Additional details of, and objections to, the experiments of Steinach and Bellesme have been presented on page 432.

The inference that storage is shown by the fact that a firefly induced to flash for prolonged periods eventually becomes unable to luminesce, is too fallacious for extended consideration.

The question of storage of photogenic material was also considered by Wielowiejski and Bongardt, though in a very circuitous fashion. Wielowiejski opposed the idea of storage of substrate on the grounds that otherwise it would be impossible to explain how fireflies are able to extinguish their light voluntarily. This idea was based on Wielowiejski's convictions that neither end-cells nor oxygen are directly concerned with the control of luminescence, and that it is inconceivable that the nervous system could affect cellular oxidations directly. To these assumptions Wielowiejski added the very dubious argument that the reason why an isolated organ can glow for a long time is that excess photogenic material is formed during the act of extirpation. Bongardt advanced what seems to be the diametrically opposed argument that photogenic material must be stored in excess, because fireflies are not able to extinguish their light suddenly. It appears, however, that both these workers had vaguely in mind the concept that luminescence continues as long as stored substrate remains, but that they differed in their ideas of how long luminescence continued and how quickly it could be terminated. Their accounts are further confused by failure to distinguish the luminescence of dead fireflies from that under normal control, and by the fact that their evidence against oxygen as a controlling factor is very equivocal.

Gerretsen's views on reactant limitation suffer from the same sort of ambiguous duality, since he assumed control by the end-cell (oxygen limitation) but, at the same time, postulated that stimulation produces a substance which emits light when it comes in contact with oxygen (control by substrate synthesis). However, Gerretsen did elaborate on the relation between synthesis and luminescence, in postulating, by analogy with Harvey's early chemical work, that the "oxidized light material" was reduced during the dark period between flashes and thus became ready for luminescence again. This idea had no experimental support until very recently, when Alexander, on the basis of his work on the effects of high oxygen tensions, postulated that luciferin "is steadily accumulating in a potentially reactive state within the photogenic cells between flashes", and that under circumstances when oxygen ceases to be limiting (*i.e.,* is present in excess), "the rate of luciferin release . . . becomes the critical factor for luminescence." Alexander, however, considered luciferin release of importance only in the hyperoxic glow and not in the control of flashing. He was unable to define precisely what should be understood by "luciferin accumulation" and "luciferin release." If either process involved synthesis, it would be hard to reconcile with continuing luminescence after death. On the other hand, if "release" were to mean a sort of reduction*, depolymerization, or hydrolysis of stored reserve, Alexander's results might be useful in interpreting long-sustained luminescences. It would, of course, have to be assumed that the "release" could continue in fireflies dried and remoistened, and that sufficient luciferin could "accumulate" prior to death to last the required time.

Space does not permit a detailed analysis of the data from which Alexander drew his conclusion. There were, however, a number of lines of evidence which fitted together consistently. On the other hand, to deal with the control of ordinary flashes and glows, Alexander adopted intact the end-cell theory of Dahlgren, which will be discussed later. It is, therefore, a possible weakness in Alexander's work that two entirely different mechanisms have to be invoked to account for all the luminescent phenomena observable in the firefly. All of Alexander's data, moreover, can be explained consistently by the modified mechanical end-cell theory which I shall present in the last section of this paper. Again, space does not permit a point-by-point comparison of the two schemes at this time. In any case, no final decision could be reached as to their respective merits, since both fit the available evidence satisfactorily. However, I consider it an advantage of the mechanical end-cell theory that it explains both normal flashing and glowing, and also the various abnormal types of luminescence.

In summary, then, there are two views in regard to the possibility that luciferin (or luciferase) could be the limiting factor in the control of luminescence. By far the most popular view is that a large excess of stored material is always present in the photogenic organ. This is supported by the common observation of long sustained luminescences, particularly in dead animals. The other view is that the substrate for luminescence is being synthesized constantly, and that there is never enough of it on hand for more than one flash. As we have seen, the evidence for this view, in the form stated, is not strong. If, on the other hand, the limitation is thought of in terms of luciferin *release,* as suggested by Alexander, rather than luciferin *synthesis,* there is a possibility of reconciling the two views, in the sense that stored reserves could exist. It should be kept in mind, however, that it would still be necessary to exclude the possibility of oxygen limitation, and to show that luciferin accumulation (synthesis) and release could explain all normal luminescent phenomena.

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* See remarks at the end of the section on the reflector layer (page 408).

Oxygen as a Possible Limiting Factor in the Control of Luminescence.* Although depriving fireflies of oxygen in various ways has been perhaps the most popular single experiment on bioluminescence, the instances are rare when this has been performed or described with sufficient precision to be of much value. To the familiar difficulties of failure to distinguish abnormal from normal luminescence, and effects on control systems from effects on luminescence *per se,* there has been added the failure to use strictly oxygen-free gases and leak-proof exposure chambers. In addition, sufficient attention often has not been paid to the facts that many common fireflies rarely flash or glow spontaneously in captivity, and that practically all fireflies are normally non-luminous by day (many observers) and in bright light (Buck, 1937a, and others). Hence, extinction of light in a given gas, or failure to luminesce, is not in itself necessarily proof that luminescence cannot occur in that gas.

In spite of these experimental deficiencies, there is no possible doubt that oxygen has a profound role in photogeny in fireflies. Luminescence has been reported to be reversibly extinguished (eventually), or not to develop, in pure N_2, H_2, or CO_2 (Forster, 1783; Spallanzani; Grotthuss, 1807; Macaire; Matteucci, 1843; Joseph; Owsjannikow, 1864; Bellesme, Dubois, Watasé, Bongardt, Townsend, Shafer, 1911; Kastle and McDermott, Creighton, Snell, Emerson, 1935; Hasama, 1942a; Alexander; Buck, 1946b). Snell's results are particularly valuable, since he was able to show that luminescence is possible below 4 mm. of O_2. This astonishingly low oxygen requirement suffices to explain reports of failure to get extinction of light with N_2, H_2 or CO_2 (Davy, 1810; Macartney, Bongardt, Kastle and McDermott). It also accounts for continued luminescence under oil (Carradori) or boiled water (Dubois), although utilization of air trapped in the tracheae may also have been involved (see p. 444). It also suggests that a control mechanism which operated by regulating oxygen would have to be of extraordinary efficiency. Additional indication of the necessity for oxygen is furnished by the observation that luminescence disappears in an evacuated space (Macaire, Owsjannikow, Dubois, Bongardt, Snell, Alexander). Here, the failures of Carradori, and of Kastle and McDermott, can be ascribed to insufficient pressure reduction, as can Dubois' claim that a dried organ glows in a vacuum if moistened.*

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* In this paper, "anaerobic" and "anoxic" will be used in their strict meanings of absence of oxygen, while "hypoxic" will be used for tensions (partial pressures) of oxygen (pO_2) which are abnormally low.

Macaire made the interesting observation that fireflies made non-luminous in a vacuum cannot be induced to luminesce, by subjection to either heat or electricity. Similar observations were made later by Bellesme and by Knoche (1910) in regard to electrical stimulation of fireflies rendered non-luminous in irrespirable gases. These experiments have been widely interpreted as proving, first, that there can be no direct nervous stimulation of the photogenic cell; and, second, that the normal control of luminescence is by way of oxygen. Since these are very important conclusions, it is necessary to emphasize that the abovementioned experiments are entirely vitiated by the probability of an anoxic effect upon the nerves themselves. In this connection, it is interesting to note that the voluntary control mechanism is usually inactivated by oxygen lack before luminescence is abolished (see pp. 444 *et seq.*). Moreover, in regard to the relevance of these experiments to direct nerve action, there is no reason to expect that luminescence could occur in the absence of oxygen.

Arnold (1881) claimed that electrical stimulation induced *L. noctiluca* to light in "absolutely oxygen-free hydrogen" (as judged by the disappearance of the glow of stick phosphorus in the same chamber). However, since (a) he stimulated only within five minutes of the darkening of the phosphorus, (b) the glow persisted for a time after the current was broken, and (c) he could get only a single response, the glowing was probably made possible by air still remaining in the interior of the tracheal system, as suggested by Edwards (1863).

Experiments with increased oxygen have also been tried, but with conflicting results. Augmented luminescence has been observed by Forster, Spallanzani, Matteucci, Owsjannikow, Bellesme, Severn (1881), Kuhnt, and Emerson and Emerson (1941), no change by Hermbstadt (1808), Davy, Kölliker, Macartney, and Dubois; and an actual decrease in emission by Macaire, Bongardt, and Hasama. Bongardt and Hasama claimed this last result to be a purely secondary one caused by the inhibitory effect of the actual gas current, because a stream of ordinary air likewise inhibited luminescence. However, their contention that this is also the explanation of the effects of N_2, H_2, and CO_2, is logically unsound. Moreover, Knoche, and Höllrigl, in repeating Bongardt's work, showed that when precautions were taken to remove O_2 from CO_2 and H_2 completely, no luminescence could be elicited in *Lampyris*. Knoche also found a stimulating rather than depressing effect of gas flow. I have reported a similar effect of gentle air-currents on the flashing of fireflies in flight in the laboratory (Buck, 1937a). Severn reported that air currents had no effect on luminescence. However, since inhibitory effects of strong air currents have been observed in the field by Geipel, and by myself, and in the laboratory by Dubois, a reflex mechanical inhibition of lighting, in accord with Bongardt's observation, is a possible source of error to be kept in mind in all work on gases, since their effects are almost always tested in streams. The effect of elevated oxygen tension is also seen in the increased luminescence obtained with high air pressure (Dubois, Lund, Alexander). According to Alexander, this corresponds with the effect obtained with nitrogen-oxygen mixtures at atmospheric pressure but with equivalent partial pressures of oxygen. Heinemann (1886) may have been dealing with the same phenomenon when he found that blowing air through a thoracic spiracle of *Pyrophorus* increased the light, although mechanical stimulation is a more likely explanation. None of the above effects bears on whether or not the oxygen effect is direct or indirect, since the experimental conditions may well have been stimulating to nerves as well as to photogenic tissue.

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* There are, as a matter of fact, two possible objections to results obtained with vacuum. First, if evaporation of water were fast enough, the cooling effect on the firefly might in itself inhibit luminescence. Second, if sufficient water were lost, lighting would be limited by water, rather than by oxygen. Probably neither objection is serious in short-term experiments with moderate rates of evacuation.

Kastle and McDermott, Snell, Maloeuf (1938), and Alexander, in work on the effect of increased oxygen on *Photinus pyralis* and *Photuris pennsylvanica,* showed that these fireflies may develop a steady glow, the mechanism of which will be discussed later ("hyperoxic glow").

Another line of inquiry which bears on oxygen limitation is one undertaken by Snell, at the suggestion of Harvey. Snell attempted to ascertain whether the nervous control of luminescence is direct or indirect, by recording flashes of *Photuris pennsylvanica* in different oxygen tensions. The analysis was based on two assumptions: first, that the actual luminescent reaction is independent of pO_2 above about 2 mm., as Shoup (1929) had found to be true in luminous bacteria; and second, that the control mechanism opens to the same extent and for the same period each time it is stimulated. It was then argued that, if the amount of light in the flashes were independent of oxygen tension over a wide range, it would indicate that luminescence was controlled by "removal of a phase boundary between reactants" in the photogenic cytoplasm, due to direct nervous stimulation. Dependence on oxygen, on the other hand, would indicate that an end-cell valve was operating, because a given response would admit less oxygen at low tensions and thus support less total light-emission. Snell did not actually compare total emissions, but only the maximum intensities of the flashes at various oxygen tensions. He found that there is apparently a progressive, though moderate, decrease in peak intensity between 150 and about 20 mm. oxygen. From this, he concluded that luminescence is controlled by regulation of oxygen by the end-cells.

It seems to me that Snell's conclusion is not justified. In the first place, there is no reliable evidence that the end-cell is the actual control mechanism. Secondly, since the constancy of operation of the control mechanism is a pure assumption, it could as well be argued that the results were caused by a slight progressive change in the control mechanism, such as might be induced in the nervous system by the changing pO_2. As a matter of fact, since Snell observed only about a 40 per cent decrease in average maximum intensity over a 7-fold change in pO_2, there must have been very considerable compensatory changes in the response of the "end-cells", if luminescence *per se* is to be considered as having been influenced by oxygen in the range from 150 to 20 mm. In general, then, the only conclusion justified from this work is that the intensity of luminescence varies with oxygen tension in the range explored. Direct nerve action is by no means excluded.

In summary, most of the careful work agrees in making oxygen essential for continued luminescence. Under various experimental conditions, oxygen may be the limiting factor in the control of photogeny, but there is no direct evidence that oxygen is ever limiting in normal flashing. Nevertheless, there is sufficient circumstantial evidence of normal control by oxygen to justify the additional inquiries described on the following pages. Thus far, the evidence has not permitted a decision as to whether the presumed control comes about by an actual limitation of oxygen as a reactant, or secondarily by the effect of oxygen limitation on some other mechanism such as the nervous system.

The Spiracular Factor in Luminescence Control. Since the spiracles guard the primary site of entry of oxygen into the tracheal system, and since, in many insects, they are known to be efficient in barring access of gases or vapors (Wigglesworth, 1939; Hoskins), their possible influence on luminescence in the firefly invites investigation. Maloeuf and Alexander mentioned the spiracles briefly in connection with their theories, but apparently they made no observations. On the other hand, Wielowiejski, Lund, and Gerretsen all argued that no control located in the large tracheae could account for the rapidity with which a flash starts and stops, because of the time required to exhaust the oxygen in the tracheal system distal to the valve. In agreement with this view, I found no correlation between the state of the spiracles and the time of occurrence or characteristics of normal or electrically induced flashes in *Photinus pyralis* (Buck, 1946b).

In the same note, I reported investigations on the influence of the spiracles on glows produced by anoxia, ether, cyanide, distilled water, and adrenalin. Injected water caused immediate spiracular closure in both normal fireflies and those glowing from ether or cyanide, and subsequent cessation of glow in the latter. With all the other treatments, the spiracles opened before glowing began, and in anoxia they closed after the glow ceased. They usually also closed at about the time the glow ceased in ether or cyanide vapor, or with injected adrenalin, but not infrequently the glow continued for a few minutes after the spiracles had closed. This was construed as showing that an "internal control" was still "open." Calculations based on measurements of the tracheae immediately associated with the light organ showed that a sufficient volume of air is trapped in and close to the light organ to support luminescence for several minutes, if the oxygen requirements of the light organ are comparable to those of ordinary tissues.

There is, apparently, a further stage of spiracular opening following the secondary closure, because dead or moribund fireflies usually have their spiracles open, whether glowing or not.

From this work, it was concluded that, although the spiracles and "internal control" often operate simultaneously, the spiracles do not ordinarily exercise any direct control over induced glows in flashing types of fireflies. However, the calculations indicate that they can terminate glowing eventually, and I think it not unlikely that they are important in luminescence control in normally glowing types of fireflies such as *Diphotus.* I attempted to investigate this question in the larva of *Photuris pennsylvanica,* but found the spiracles too small.

As argued in regard to breathing movements, if the spiracles were the only means of oxygen control for the organism, luminescence would be expected to occur by day, since the spiracles would presumably have to open to meet respiratory needs. This would not agree with the usual reports that fireflies are non-luminous by day. However, since fireflies are also inactive by day, it is possible that their general metabolism falls so low that the spiracles would need to be opened only very infrequently. Under such circumstances, even if luminescence occurred when the spiracles opened, it might escape observation.

The Internal Control of Glowing. A large number of agents and treatments have been discovered which cause glowing in Type 6 fireflies (Verworn, Kastle and McDermott, Gerretsen, Creighton, Snell, Emerson, Emerson and Emerson, Alexander; Seifter, 1945; etc.). Of these, hypoxia and anesthetic vapors have proved to be particularly useful in studying the operation of the control mechanism, and from their use a number of conclusions have been drawn concerning the tracheal end-cell. Since none of this work actually bears on the anatomical identity of the regulatory mechanism, I shall use the noncommital term, *internal control.*

Verworn, and also Gerretsen, studied the effect of strong chloroform vapor on *Luciola italica* and *Luciola vittata,* respectively, forms which apparently give frequent spontaneous flashes under laboratory conditions, in contrast to the rare flashing of our American *Photinus pyralis.* They observed three stages of chloroform effect: (1) reversible inhibition of flashing, with immobilization, and sometimes accompanied by a dull glow; (2) irreversible bright glowing; (3) irreversible extinction. Stage 1 was ascribed by Verworn to nerve narcosis, and by Gerretsen to transient contraction of the end-cells. Stage 2 was assigned by Verworn to direct chemical stimulation of the nerves and photogenic tissue, and by Gerretsen to relaxation of the end-cells. Stage 3 was attributed by Verworn to exhaustion of the stored photogenic material, due to the luminescence in the second stage, and by Gerretsen to permanent damage to enzymes. Verworn's opinion in regard to Stage 3 is weakened by Gerretsen's observation that the dead fireflies in Stage 2 sometimes glowed for hours if removed from the chloroform vapor. The idea of some sort of irreversible chemical damage to the luminescent system is supported also by my observations on *Photuris pennsylvanica.* In this species, I found that light organs from specimens rendered non-luminous by an hour's exposure to saturated ether vapor, would not glow even if minced in fresh air, a treatment which ordinarily suffices to cause glowing even in moribund animals. The above idea is also supported indirectly by Emerson's work with known concentrations of ether, in which he found that irreversible damage to the luminescent system results when lethal concentrations are reached.

Either because they used saturated vapor or because chloroform is rather toxic, Verworn and Gerretsen missed some important intermediate stages of anesthesia which can be demonstrated in gradual hypoxia and with gradually increasing concentrations of ether. Thus, Emerson, and also Buck (1946b) have shown that bright early ether glows are readily reversible. The same is true for glows produced by low oxygen (Bongardt, Kastle and McDermott, Snell, Alexander; Buck, 1946b).

Snell studied the effect of low oxygen tension carefully, and showed not only that partial pressures below 4 mm. induce a steady dull hypoxic ("anoxic") glow[*] but also that, if the oxygen tension is suddenly raised from below 4 mm. to 30 mm. or above, the glow brightens suddenly, and usually markedly, and then quickly subsides to zero ("pseudoflash"). Snell interpreted the hypoxic glow as due to entry of oxygen through inactivated end-cells, and the pseudoflash as an extra uncontrolled luminescence which is terminated by recovery and closure of the control mechanism (end-cell valve).[†]

A further refinement in "internal control" action was disclosed by my finding (Buck, 1946b) that with very gradually decreasing pO_2 a stage is reached where an hypoxic glow has not yet developed, but in which a pseudoflash can be induced. To explain this phenomenon, I postulated that the "aperture" of the internal control had enlarged enough to permit luminescence at high pO_2 (pseudoflash), but still not enough to permit it at low. Since luminescence (hypoxic glow) does develop at oxygen tensions still lower than in the above stage, I concluded that the aperture of the internal control enlarges faster than the pO_2 decreases. In other words, the internal control overcompensates in its response to decreasing oxygen tension. These facts enable us to make the further deductions that luminescence requires the entry of a certain minimum number of oxygen molecules per unit time, and that this can be achieved either by the entry of air higher in oxygen through a smaller aperture, or by the entry of air lower in oxygen through a larger aperture. This implies, furthermore, that the response of the internal control is a graded, rather than all-or-none phenomenon.

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[*] The hypoxic glow is ordinarily produced by using pure N_2, H_2 or CO_2, but Bongardt, Snell, and Alexander showed that it occurs also in atmospheric air if the total pressure is lowered enough to decrease pO_2 below 4 mm.

[†] The pseudoflash has been interpreted by Alexander in terms of the Wigglesworth-Maloeuf osmotic theory, which will be discussed later. It might also represent the oxidation of luciferin that had accumulated during the hypoxic period, as has been shown to occur in the "flash" of luminous bacteria. However, in a number of experiments with *Photinus pyralis,* I found no correlation between the durations of pseudoflashes and the durations of the preceding anoxic periods.

Further information on the operation of the "internal control" is contained in a number of observations bearing on whether or not flashing and glowing are controlled by the same mechanism. It has been reported generally that normal flashing does not occur under anesthesia, except occasionally at the start of exposure, before glowing has developed to full intensity. Unfortunately, the evidence is not clear-cut on whether or not flashing can be elicited artificially during glowing. Although his account is very ambiguous, Gerretsen apparently believed that luminescence could be affected by electrical shock during his Stage 2 (glowing) of chloroform narcosis. On the other hand, Snell (1932) and Alexander found that normal flashing is inhibited during the hypoxic glow. Furthermore, Alexander was unable to induce flashing during hypoxic glowing, by electrical treatment. These results have been regarded by Alexander, and others, as showing that the flash control cannot operate during glowing, or in other words, that flashing and glowing are independent phenomena. However, none of this work circumvents the objection that that hypoxia and anesthesia (like anoxia) undoubtedly abolish the normal functioning of the flash control mechanism. Furthermore, the failure to obtain flashing with hypoxia might have been due simply to the very low pO_2, rather than to a necessary difference in the control mechanisms for flashing and glowing. In other words, luminescence may already have been maximal for the existing pO_2.

Snell's work with low oxygen is particularly germane with regard to the distinction between glow and flash. He found that, below about 20 mm. oxygen tension, the duration of the flash lengthened progressively and the peak intensity decreased until, at 4 mm., the steady hypoxic glow resulted. The region between 20 and 4 mm. oxygen tension, therefore, is a transition territory in which it is hardly possible to distinguish flash from glow.

An additional point of importance concerns the normal "neuromuscular" state of the internal control mechanism. The fact that fireflies moribund from inanition (or those which fail to recover from moderate anesthesia) often exhibit a steady, lasting glow, indicates that in its relaxed position the "valve" is open, and that energy expenditure is required to keep it closed. The same conclusion is indicated by the work of Gerretsen and of Emerson on anesthetics, and by the work on the effects of oxygen, if the assumption is made that the internal control relaxes (opens) in low oxygen and contracts (shuts) in high (Snell, Alexander; Buck, 1946b). As a matter of fact, adrenalin, cyanide, and all the other glow-inducing agents can be imagined to have an analogous effect. The open position in hypoxia, anoxia, anesthesia, and death is compatible with an assumption that energy expenditure is required to keep the "valve" in the closed (tonic), or non-luminescent, condition. This point will be discussed further on page 450.

A further interesting point was discovered by Alexander in his extension of Snell's work on the effects of high oxygen tensions. Although there is not space here to give the evidence in detail, Alexander was led to conclude that the glow produced by high oxygen is due to oxygen leaking directly through the cytoplasm, thus by-passing the internal control.

In experiments on glows produced by ether, carbon dioxide, and adrenalin, I found the responses of *Photuris pennsylvanica* larvae similar to those of the adult *Photinus pyralis.* Since the larva lacks end-cells, it can be concluded that end-cells are not necessarily involved in the control of glow in adult fireflies. However, they might still be essential to flash control, since none of the work discussed in this section really touches that point.

Theoretical Mechanisms of Luminescence Control by Oxygen Limitation:

INTRODUCTION. In this section, the mechanisms of oxygen control which have been proposed in sufficient detail will be analyzed in the light of the anatomical and physiological evidence discussed, and according to certain theoretical considerations. A review of the anatomical path which oxygen must follow in its journey to the photogenic cells, suggests the following sites at which the passage of oxygen might be interrupted: the spiracle, the end-cell, the tracheole, the differentiated zone, the membrane of the photogenic cell, and the photogenic cytoplasm. The spiracle has already been considered and found not to be concerned in the control of flashing. What little information exists on the differentiated zone of the photogenic cytoplasm, and on the possibility of phase-boundary changes in the cytoplasm, has been presented and need not be reconsidered. The membrane of the photogenic cell is theoretically the most logical site of all for the operation of an efficient control, but since Harvey (1922) has shown that a number of cells are very permeable to oxygen, there is no reason to expect that control by changes in permeability to oxygen is likely.* We are reduced, therefore, to the tracheoles and the end-cells as possible agents in oxygen control.

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* There is every reason to believe that this conclusion is valid generally, but Harvey's work actually concerned permeability under abnormal conditions (recovery from anoxia).

The discussion will be mainly in terms of the Type 6 organ, since it has been the subject of nearly all the modern physiological work. However, this should not justify any generalizations, either pro or con, concerning any particular theory. Thus, it seems certain from the time relations, if from nothing else, that a firefly with an intermittently glowing type of organ will require a very much less precise and intricate control mechanism than one of Type 6.

COMPETITION FOR OXYGEN, IN RELATION TO CONTROL. One question which may have an important bearing on control is whether or not there is competition for oxygen between respiration and luminescence. This possibility exists because both systems presumably draw their oxygen from a common intracellular "pool".[*]

There would be potential competition if, during luminescence, the oxygen supply fell below that necessary to support both luminescence and basal respiration. There are few data which bear, even indirectly, on what occurs in these abnormal circumstances. Harvey (1922) found that, in *Chaetopterus* sealed in water, luminescence disappeared long before spontaneous muscular activity. However this may not indicate that luminescence is more sensitive to oxygen lack than is respiration, but only that the oxygen uptake necessary to complete the enzymatic reactions in muscle can be long delayed, whereas those in luminescence cannot. Moreover, it is possible that the effect was due to accumulation of carbon dioxide, rather than to deprivation of oxygen. Shoup found that respiration[†] of luminous bacteria began to fall off when pO_2 had been reduced below 23 mm., whereas luminous intensity was unaffected until pO_2 reached 2 mm., at which point respiration was only 50 per cent of normal. If, as seems reasonable, the intensity of luminescence is proportional to the oxygen consumption of the luminescent reaction, these results suggest that luminescence may be more successful in obtaining oxygen from a deficient supply than is respiration. It is, however, not certain that this conclusion is applicable to fireflies.

If, as Shoup's results suggest, luminescence has a competitive advantage over respiration under conditions of restricted oxygen, it appears unlikely that luminescence could be suppressed by respiration under normal circumstances, where sufficient oxygen for respiration is always present. However, since Creighton has proposed such a mechanism, it will be worthwhile to analyze it in some detail, particularly as the analysis points to a possible experimental test.

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[*] Recent work on luminous bacteria indicates that, although respiration and luminescence show considerably different responses to certain stimulating and inhibiting agents (*e.g.*, potassium cyanide and urethane), they utilize parallel enzymatic pathways. With increased oxygen, for example, proportionately more oxygen goes through the luminescent system. (See Harvey, 1940, 1941, and Johnson et al.)

[†] Shoup used "respiration" as synonymous with total oxygen uptake, but in the present paper it is used in the restricted sense of oxygen uptake other than that required for luminescence. In practice, the two measures will probably be nearly identical, since Shoup's work, and that of Snell on the hypoxic glow of the firefly, indicate that the oxygen requirement of luminescence is very small in comparison with that of respiration.

In view of Shoup's work, respiration could deprive luminescence of oxygen only under one of the following conditions: (a) the oxygen requirements of respiration are actually lower than those of luminescence, and the photogenic tissue is maintained nearly anaerobic, at least during the dark periods; (b) respiratory activity is localized in a cortex surrounding the actual photogenic cytoplasm, so that oxygen diffusing in it can be "filtered" out before reaching the other reactions of luminescence; (c) the intracellular oxygen transport system channels oxygen preferentially into respiration (at normal pO_2). The first possibility is rendered very unlikely by the known low oxygen requirement of luminescence. The second possibility certainly appears unreasonable on general grounds. However, in its light, Dahlgren's speculations on the "oxygen-insulating" properties of the differentiated layer of the photogenic cytoplasm (see p. 413) assume a new and provocative significance, and perhaps deserve further consideration. The third condition is one which, by analogy with known systems, is possible, but which would not be expected to permit absolute extinction of luminescence,* since such "channelings" are usually questions of relative rates of utilization, rather than absolute exclusion.

Since the idea of competition seems not impossible, an enumeration of its nature and consequences is in order. According to Creighton's hypothesis, basal respiration normally uses all the oxygen available and, hence, prevents visible luminescence (dark period). If, however, additional oxygen is made available (*e.g.*, by end-cell contraction), photogeny may begin (luminescent period). There is, however, an alternative method by which competition could operate. According to this second hypothesis, the oxygen supply remains relatively constant, but is usually all appropriated by respiration, which proceeds at a rate higher than basal (dark period). If, however, the respiratory rate falls, oxygen is available for the luminescent reaction. Both hypotheses, it will be seen, are compatible with the fact that respiration can be maintained without permitting photogeny at "inappropriate" times. This is particularly pertinent in view of the very small pO_2 required for luminescence. Both hypotheses also are in harmony with the expectation that respiration is diminished or absent in moribund or dead fireflies, where luminescence is often continuous. Assuming that prolonged anesthesia depresses respiration, both hypotheses also explain glowing in narcosis. Neither hypothesis accounts for either hyperoxic or hypoxic glowing, without further assumptions which are too detailed to consider here.

Theoretically, a decision as to which mechanism (if either) is operating could be made by comparing the oxygen uptakes of a tissue in the dark and in the luminescent states. According to the first hypothesis, oxygen consumption should be higher during luminescence than during the dark period; according to the second, probably lower.* Unfortunately, as already discussed, no direct data are available.† However, it will be instructive to pursue some of the consequences of the two hypotheses a little further.

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* In view of the extreme sensitivity of the human eye, it is likely that luminescence really is zero during the apparently "dark" period, rather than continuing at a low rate.

The first hypothesis, according to which respiration is lowest during the dark period, fits better the traditional idea that control is achieved by limitation of the external oxygen supply. It agrees, for example, with the expectation that luminescence involves increased oxygen and that nervous stimulation induces luminescence by inducing an increase in that supply. Furthermore, a linear relation between luminescence and oxygen tension, at least over part of the range, would be expected on the basis of the first hypothesis, if the oxygen uptake of luminescence is linear with oxygen tension. No relevant work has been done on glowing forms, which would be the most favorable for the purpose; however, as we have seen, Snell's data indicated that peak flash intensity decreases as the partial pressure of oxygen decreases from atmospheric. On the other hand, in several organisms (notably bacteria), luminescence has been found to be independent of oxygen over very wide ranges.

The second hypothesis, in common with other intracellular controls, has the advantage over Creighton's mechanism of potentially greater speed, and the apparent "disadvantage" of not accounting for the end-cell. In addition, it requires us to think of nervous "stimulation" of luminescence in terms of inhibition of respiration, and of the firefly as expending more energy in keeping itself dark than in luminescing.

On the whole, although a competitive mechanism is not excluded from being concerned in firefly luminescence, the probability does not seem large enough to justify pursuing the question further. Likewise, in the absence of experimental work, there seems to be little point in attempting to choose between Creighton's hypothesis and its alternative.

The Theory of Osmotic Control of Luminescence. In 1930, Wigglesworth proposed an ingenious theory, according to which the changing needs of a cell for oxygen could be met by varying the amount of fluid in the distal end of the tracheole. This theory was not originally designed for the special anatomy of the firefly, with its end-cells and anastomosing tracheoles and, moreover, it assumed intracellular penetration of the tracheoles. Several years later, however, it was applied specifically to the firefly by Maloeuf.

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* It is generally assumed that the oxygen consumption of the luminescent reaction occurs at the same time the light is emitted. This appears to be supported by the fact that oxygen is essential for continued luminescence. However, over periods as short as those of normal flashes, it might well be that light-emission and oxygen uptake would not be simultaneous. An analogy is offered by muscle, in which the oxygen is used in the recovery or preparatory period, whereas the actual reactions involved in contraction occur anaerobically. Therefore, a comparison of oxygen uptakes during light and dark states of a tissue might not give a true measure of the extra oxygen consumption involved in the luminescent reaction.

† Some idea of the oxygen requirements of the same tissue in the luminous and non-luminous states might be obtained by comparing quiescent normal fireflies with brightly glowing "dead" specimens, or, perhaps (to eliminate the possibility of oxygen uptake by surviving tissues or by bacteria), with fireflies which had been dried and then remoistened. This would not, however, be ideal, because basal respiration would be lacking in the dead group. It may be of interest, also, to mention here my preliminary experiments on the oxygen uptake of specimens of *Photinus pyralis* poisoned with DDT. As with other insects, oxygen uptake was increased several-fold. Luminescence was also, at first, increased over the near inactivity usual in the laboratory, but consisted of irregular flashing and intermittent glowing, suggestive of nerve irritation. It was only late in the experiment, when muscular activity and oxygen uptake had both declined, that luminescence became continuous.

Wigglesworth proposed, first, that the tracheolar wall is semi-permeable. Second, he postulated that the level to which air extends in the tracheoles is determined by a balance between the osmotic pressure of the cytoplasm (which tends to pull water out of the tracheole into the cell) and the capillary attraction of the liquid in the tube (which tends to draw water out of the cell). He supported this idea by showing that hypertonic solutions injected into mosquito larvae caused movements of the air in the tracheoles. He further assumed that, if the osmotic pressure into the cell were increased during cell activity, as by the breaking-down of large substrate molecules into smaller metabolic products such as lactic acid, water would be withdrawn from the tracheole into the cell. This would allow the air to extend further in toward the cell and meet the increased need for oxygen imposed by the increased activity. Conversely, during quiescence, materials would be resynthesized in the cytoplasm, causing its osmotic pressure to fall, and water would pass from the cell into the tracheole and force the oxygen supply farther away. In agreement with expectation, Wigglesworth was able to show reversible inward movement of air, and acid formation, during anoxia. Maloeuf's contribution consisted in defining the "activity" which increases the osmotic pressure, as the luminescence of the photogenic cell, and in showing that injection of hypertonic and hypotonic solutions into the photogenic organ caused, respectively, stimulation and suppression of luminescence. Dubois, however, had found that water injection induced glowing.

The osmotic control theory has run into several difficulties. First, Wigglesworth (1938 a and b) found, in developing mosquito larvae, a number of phenomena which were not explicable by his theory in its original form. Shortly thereafter, Bult (1939) introduced a considerably altered version of the theory and, by processes too complex to elaborate here, came to the conclusion that an increase in osmotic pressure in the cell would actually bring about movement of air *away* from the cell. The "osmotic" effects he therefore attributed to swelling of cell proteins. The Bult theory could probably be adapted to the problem of photogenic control, although it is by no means clear that it is a substantial improvement over the original Wigglesworth theory. Both theories, however, suffer from the serious drawback that the observed speed of the fluid movements produced is very slow. Over distances comparable to those met in the tracheoles of the light organ, the time required is of the order of minutes, where seconds or fractions of seconds are required.

Alexander repeated Maloeuf's experiments in detail and confirmed the findings in regard to hypertonic solutions, but not concerning hypotonic solutions. In some instances, moreover, he found that the direction of spread of glow in the organ did not correspond with that expected by theory. He concluded that tracheolar fluid movements are not concerned in normal flashing, although they probably are in the glow caused by injected hypertonic solutions, and in the fact that the pseudoflash has a shorter latent period than has hyperoxic glow. Since we have already seen that many substances beside hypertonic solutions induce glowing, this effect cannot safely be ascribed to osmotic changes. Likewise, as will be shown shortly, the length of the latent period between stimulus and light-production can be explained adequately by a valve mechanism which opens in low oxygen tensions and closes in high. Another explanation of the effect of fluid in the tracheoles will also be presented.

The Mechanical Valve Theory of End-Cell Control of Oxygen. By far the majority of theories concerning the end-cell give it the role of a regulator of oxygen rather than a source or adjunct of luminescence. Most of these theories are in terms of the generalities which have already been discussed, as, *e.g.*, the strategic location of the end-cell, its supposed innervation, and especially its presence in flashing fireflies and absence in glowing ones.* This last point seems to me to be a particularly strong one, though, admittedly, it provides no information on how the end-cell works. The objection which is often raised that end-cells are found in non-luminous tissues, even in the firefly, does not show that they cannot operate in regulating oxygen. Schultze has pointed out that it is not the exclusive possession of end-cells, but their strikingly high concentration, which distinguishes the photogenic layer from other tissues.

Dahlgren and most other writers on end-cell physiology have assumed that the tracheoles are at least partly air-filled. As we have seen, the evidence is by no means unequivocal in this respect, but unless it is assumed that an actual current of liquid is circulated through the tracheoles, we shall have to postulate the presence of gas distal to the end-cell, as well as proximal to it, if the end-cell is to operate as a valve. This is because the speed of diffusion of oxygen through tissue is not greatly different from that through plain water (Krogh), so that, if the tracheoles were water-filled, the end-cell would offer relatively little hindrance to aqueous diffusion.

Though a number of investigators have thought of the end-cell in frankly physical terms, Dahlgren is almost alone in describing structures which might make possible the valvular action. As already mentioned, he found, in osmic acid preparations, that the tracheal twig narrows within the end-cell, loses its spiral thickenings, and shows an annular "dark-staining sheath", "specialized body", "cylindrical organ" or

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* **Maloeuf** has made much of his claim that **Bongardt** found end-cells in the larva of *Phosphaenus hemipterus*, an animal which, according to **Maloeuf**, "displays no such brisk flashing rhythm." I would not regard this as conclusive in any case, in view of the contradictions in **Bongardt's** paper but, actually, **Bongardt** (p. 24) says that end-cells are absent.

"rounded mass" ("S" in FIGURE 11).* This staining reaction, continues Dahlgren, "may point to a contractile layer of cytoplasm surrounding it, with the possibility of a valve, in addition, in its lumen. Also the larger body of cytoplasm surrounding it sometimes shows a radial structure that may point to a general contractile power."†

Dahlgren explains the functioning of the end-cell as follows: "It is believed by the writer that these radiating rods and the rounded mass represent muscular structures which are of two possible uses: to prevent the passage of air into the tracheole by compressing them; this would appear to be the function of the rounded mass; second, to enlarge the terminal twig and end organ and thus fill it with a new supply of air; this would seem to be a possible function of the radiating rods. It can be seen that the rapid alternation of the two actions would result in the forcing of a sudden jet of air bearing free oxygen into the light cell mass, and would account for the power of flashing exhibited by the organ." Dahlgren suggests, in addition, that two sorts of nerves will be required, one for the rounded body and one for the fibrillar cytoplasm. His hypothesis was used uncritically by Creighton, Snell, and Alexander in their physiological work. Alexander, in fact, interprets most of his results on the basis of end-cells able to cause a "sudden burst of oxygen directly into the photogenic cells".

It seems to me that Dahlgren's mechanism, in the form stated, is equivalent to pinching a tapering tube open at both ends, and expecting the jet of air to move toward the smaller end (that is, into the tracheoles). Matters would be even worse if the small end of the tube were partly or entirely closed, as it might be if the tracheole contained some water or terminated intracellularly. In order to enable Dahlgren's end-cell to actually produce a "jet" of air, several additional mechanical properties must be postulated for it, and a number of new assumptions introduced. Since these requirements apply to any mechanical end-cell valve theory, it is worth while to enumerate them, as follows.

(a) The "rounded mass" must lie proximal to the region of the end-cell lumen which is enlarged by contraction of the fibrillar protoplasm. This is necessary in order that the contraction of the rounded body can shut one end of the tube through the end-cell, so as to allow pressure to build up distally.‡

(b) The passage through the end-cell must have an elastic wall, in order to provide a means of applying pressure to the air which is drawn into the end-cell when the passage is enlarged by contraction of the fibrillar cytoplasm.

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* Dahlgren seems to be somewhat vague about the exact location of this body, since in his Figure 11 (p. 611) he shows it as indicated in the lower of the two end-cells pictured in my FIGURE 11, whereas in another version (his Figure 20, p. 345), diagrammed as the upper of the two end-cells in my FIGURE 11, it does not seem to appear.

† I, too, have seen a striated appearance in the end-cell cytoplasm in some preparations (Buck, 1940), but the structures are not very sharp. They, and indeed the whole end-cell, are very small.

‡ Dr. H. Specht has pointed out to me that the internal surface of the end-cell lumen must be kept dry, since otherwise a meniscus would form at the point of constriction, and resist, with relatively enormous force, the reopening of the tubule.

(c) The periphery of the end-cell must be rigid. This will be necessary in order to give the "fibers" something to anchor to as they pull on the wall of the lumen of the end-cell. As already mentioned, the periphery of the end-cell is notably lacking in any suggestion of such a compact structure.

(d) By analogy with vertebrate sphincters, the "rounded body" ought to be composed of two layers of contractile elements at right angles to one another, one for enlarging its opening and one for constriction.*

(e) In the relaxed state of the fibrillar protoplasm, the part of the end-cell lumen which it controls must still be patent.

With these new specifications, we are in a position to revise Dahlgren's account of the operation of the end-cell. This will now require four successive stages, as follows.

First: Contraction of the fibrillar cytoplasm to enlarge the lumen of the end-cell and draw air in from the terminal tracheal twig.

Second: Contraction of the rounded mass to close off the end-cell lumen proximal to the point enlarged by the contraction of the fibrillar protoplasm.

Third: Relaxation of the fibrillar cytoplasm, so that the elastic recoil of the wall of the end-cell lumen applies pressure to the contained air and forces some of it distally into the tracheoles.

Fourth: Relaxation of the rounded mass, opening its lumen and releasing the pressure on the air in the tracheoles. Since the entire passage through the end-cell is now open, diffusion can replenish the oxygen in the tracheolar air.

Since the anatomical and operational features just outlined are so largely speculative, it is desirable to ascertain how well they meet criticisms which have been made of the concept of a mechanical end-cell valve, and how well they explain various known facts about the control mechanism.

Maloeuf raised several objections, based on his understanding that Dahlgren's theory provided for *stopping* the flash by contraction of the end-cell. This is another of the points lacking in Dahlgren's exposition. According to the revised mechanism outlined above, the decay of luminescence would be caused simply by the decrease in pO_2 due to respiration and luminescence, to a level insufficient to support luminescence. The flash is actually *started,* rather than stopped, by end-cell contraction.

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* If such intricacy seems difficult to visualize in a structure as small as an end-cell, we should recall the complexity of some of the small Protozoa, to say nothing of that of an individual cilium.

Another obvious question to be asked about the mechanical end-cell control theory is whether the amount of air which could be forced into the photogenic tissue in a single "jet" would be sufficient to support the necessary luminescence. We are seriously hampered here by not knowing what that requirement is, but, if the demands of light-production are anything like those of ordinary tissue respiration, it seems unlikely that the mere contraction of the sphincter-like rounded mass (resulting in the inward movement, by a distance of a micron or two at most, of a fraction of a cubic micron of air) would be adequate. The same objection would probably apply to any theory involving simple constriction at some point in the lumen of the end-cell, even allowing for the possibility that the end-cell at the other end of the tracheole might provide a similar amount of air simultaneously. Under such circumstances, it seems that a preliminary enlargement of the end-cell passage, such as Dahlgren postulated, would be necessary. On the other hand, if the oxygen requirement of luminescence were very low, it might be possible to simplify the contractile mechanism to the extent of eliminating the expansion of the tracheolar lumen caused by contraction of the fibrillar protoplasm. The mechanism would then consist simply of two sphincters in tandem on the end-cell lumen, contracting and relaxing with the usual peristaltic rhythm.

Dahlgren describes the tracheoles as intercellular, and as connecting two end-cells of contiguous cylinders (anastomosis), but he does not consider specifically whether the "jet" is to be a *flow* of air through the open tube or a raising of the air pressure inside the tracheole. The former would result if the end-cell at one end of the tracheole were relaxed while the other was contracted; the latter, if both end-cells contracted at once. The former, or "flow" hypothesis, would have the following consequences:

(a) It might result in a more efficient renewal of the tracheolar oxygen, since air low in oxygen would be exhausted into a large space (the twig) with each pulse, where diffusion could raise its oxygen content quickly.

(b) It would require a high order of nervous coordination (alternate firing of the members of end-cell or cylinder pairs).

(c) Luminescence should spread from one end-cell toward its partner.

The "pressure" hypothesis would have the following consequences:

(a) It could operate with simultaneous firing of end-cells.

(b) Action would probably be faster than with a "flow" mechanism, since, in the latter, each flash would have to progress across the photogenic cell.

(c) It might result in a higher momentary oxygen tension in the tracheole than could be obtained by flow alone.

(d) Increase in oxygen tension would be simultaneous along the whole tracheole.

It seems to me that the points enumerated illustrate certain real advantages of a pressure mechanism. It has been pointed out previously that the final step in any oxygen control mechanism must be the passage of oxygen into the photogenic cytoplasm, by diffusion. Since the rate of diffusion cannot be changed, the problem of bringing about very rapid control becomes one of making diffusion distances as short as possible. Such a requirement is obviously not met by any arrangement whereby oxygen reaches the surface of the photogenic cell by diffusing down the tracheole. Nor is it met by a flow mechanism, because time is lost in raising the oxygen tension at successive points along the tracheole. With a pressure mechanism, however, most of the necessary oxygen can already be present in the tracheole, though ineffective in causing luminescence, due to its partial pressure being sub-threshold or because all is being used in respiration (see discussion of competition hypothesis). The pressure increase, therefore, results in a simultaneous rise in oxygen tension at all points in the tracheole, at a rate limited only by the speed of contraction of the end-cell. The rate of build-up of the flash is, then, determined by the speed of gaseous diffusion over a maximum distance of the radius of the tracheole, plus liquid diffusion in the photogenic cytoplasm. In the same way, the decay phase, or shutting-off of the flash, can occur as rapidly as the end-cell can relax,* because the oxygen tension falls simultaneously to a sub-threshold value at every point in the tracheole.

Another, if less crucial, advantage of the pressure hypothesis is that it would also apply if an inner section of the tracheole were blocked or restricted by liquid or tissue. The only effect of liquid in part of the tracheole would be to reduce the intensity of the flash by impeding oxygen access to part of the photogenic cytoplasm. In fact, such fluid might even be assigned a possible function in so varying flash intensity, thus taking care of those reports which describe fluid in the tracheoles. Moreover, since fluid would not affect the duration of the flash, one explanation is provided for Snell's observation that, although the intensity of normal flashes is highly variable, the duration is astonishingly constant. It is thus one of the advantages of the mechanical end-cell theory, in the revised form presented here, that it would operate whether the tracheoles are extracellular or intracellular, or whether they do or do not anastomose (provided that, if they do, the two end-cells of a tracheole contract simultaneously).

This whole superstructure is so complex and highly theoretical that it may seem incongruous to speak of considering the "evidence" for one or the other hypothesis. Nevertheless, it must be kept in mind that the control of luminescence in the firefly, no matter how it is brought about, is, after all, a wonderfully precise accomplishment. It would thus be surprising, indeed, if its mechanism were not intricate, both anatomically and physiologically. At any rate, the observations of Lund, Alexander, and others, that luminescence spreads out from the cylinder wall, might be thought to favor the "flow" hypothesis. However, it must be remembered that all such observations were of glows rather than flashes and, as we have seen, the glow is most reasonably explained as due merely to diffusion through an inactivated control mechanism. In terms of the present theory, this "inactivation" would mean the relaxation (opening) of the rounded mass, without any activity of the fibrillar cytoplasm.

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* Since relaxation decreases pO_2 in the tracheole below the threshold for luminescence, decay is determined only by depletion of the oxygen actually in the cytoplasm. According to the revised version of the mechanical end-cell theory, the "contraction", or action which forces air into the tracheole would actually be a relaxation of the fibrillar cytoplasm, the force being provided by elastic recoil of the wall of the end-cell lumen. The "relaxation" would be the relaxation of the "rounded body."

Further important evidence on the mechanical theory of end-cell action can be derived from considering whether it can also explain the phenomena discussed in connection with glowing. Alexander strongly emphasized the distinction between glow control and flash control, and apparently thought of the two as being quite separate, although most of his conclusions on flash control were derived from observations of glows. The points which particularly seem to indicate a dual control are the following:

(a) Voluntary flashing is abolished in the dead or decapitated animal, whereas glowing is often brilliant.

(b) Normal or electrically stimulated flashes usually occur independently of glowing.

(c) Voluntary flashing is abolished during glows produced by anesthetics or by high or low oxygen. During the latter, at least, flashing cannot even be elicited by electrical stimulation.

(d) No glow approaches in intensity the maximum reached in the flash.

(e) Under some circumstances, flashes can occur superimposed on a glow (early stages of high oxygen effect—Alexander; early stages of anesthesia or water injection—Buck).

(f) The decay portion of the pseudoflash time-intensity curve is far more gradual than that of the normal flash (Snell, Alexander).

All these observations can be explained as follows, on the basis of a valvular end-cell which has the properties which I have postulated:

(a) In the normal non-luminescent condition, the rounded body is in a state of tonic contraction such that its lumen admits only enough air to supply respiration, and the fibrillar protoplasm is relaxed, so that the part of the lumen it controls is minimal.

(b) In response to voluntary or to applied electrical stimulus, there occurs the four-stage contraction-relaxation sequence postulated as producing the air "jet." At the end of the resultant flash, the rounded body returns to its original state, with an opening insufficient to permit glow. (Flash without glow.)

(c) Anesthetics or death relax the rounded body enough so that sufficient air diffuses through to exceed the oxygen threshold for glowing. Or, alternatively, these factors depress respiration, so that oxygen becomes available for photogeny. They also inactivate the fibrillar cytoplasm, which is already relaxed (open), so that no flash is possible. (Glow without flash.)

(d) The assumption that, in ordinary air at one atmosphere pressure, glow intensity is determined only by the aperture through the rounded body, would explain why the glow (diffusion-controlled) is not as intense as the flash (pressure-controlled).

(e) A slight stimulation, preceding anesthetic or toxic effect, is a well-known pharmacological phenomenon. A slight stimulation of the fibrillar protoplasm of the end-cell, at the same time that the rounded body reached its relaxed phase, would well explain flashes superimposed on glows.*

(f) The slow decay of the pseudoflash, as compared with that of the normal flash, can be explained by a slowed-down response of the rounded body as it recovers from anoxia and resumes its tonic state of partial contraction.

In summary, the facts and assumptions presented in the last few pages suffice to explain many of the known facts about light-emission in the firefly, on the basis of mechanical control of oxygen by the end-cell. There is, however, no strong evidence that the concept is more than a hypothesis, except the correlation between the possession of end-cells and the ability to flash. Moreover, there is no proof that oxygen limitation operates directly to control luminescence. On the other hand, there exists also the hypothesis of direct nerve action, which explains known phenomena as satisfactorily as the end-cell theory. This hypothesis is supported by the fact that intensity of luminescence varies to some extent with intensity of stimulation in forms without end-cells, and by a variety of other suggestive but not "air-tight" experiments, particularly those of Bellesme, Steinach, and Snell. An obvious question is whether there is any possibility of reconciling the two ideas, so that the presence of end-cells is explicable while the apparent physiological advantages of direct nerve action are retained.

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* As an additional refinement, it could be assumed that the rounded body may also contract (close) initially in response to a brief stimulation by anesthetics. This would explain Gerretsen's observation of an initial transitory suppression of luminescence by anesthetics, which Maloeuf used as an argument against the mechanical control of flashing.

An ostensible reconciliation was achieved in Brücke's (1881) suggestion that oxygen exercises control indirectly, no luminescence occurring when the direct nerves to the photogenic tissue are kept anaerobic. There is little anatomical support for this idea, nor is it easy to reconcile with, *e.g.*, the hypoxic glow. Furthermore, it apparently requires a second set of "indirect" nerves for regulating the oxygen (*e.g.*, *via* end-cells). However, the possibility of reconciling the two major hypotheses of control is so alluring that it is to be hoped that some further evidence will be adduced which points to an "indirect" function of the end-cell.

On the whole, the strongest argument against direct control of luminescence by oxygen is the fact that no other similar mechanism is known. It is true that rapid metabolic responses are, as yet, very imperfectly understood, but in the few instances which have been at least partially worked out (nerve, muscle, activation of dormant respiration), all the evidence points to enzymatic activity as the physiological "trigger" which sets off the reaction. Such enzymatic activity, in other words, appears to be the usual method of bringing about metabolic changes which are sudden and complete. There is no point in attempting to propose any specific mechanism in regard to control of luminescence, while the analogous control of muscular contraction is still unclear after an immense amount of good experimental work. However, it can be anticipated that the study of enzymatic inhibition and activation in the luminescent system will prove to be a fruitful field in future research on the control problem.

SUMMARY

In spite of the many morphological and physiological data which concern luminescence in the firefly, there seem to be surprisingly few unequivocal major conclusions which can be drawn. This is due partly to the many differences which exist between various species of fireflies and which often make generalizations impossible, and partly to the fact that our knowledge is seriously deficient in many essential points. Among the generalizations which can be made are the following:

1. The luminous organs of fireflies vary greatly in size, shape and position, and with sex and developmental stage.

2. Firefly light organs can be divided into six histological types: (1) those with no specific tracheal supply; (2) those in which the tracheae show tree-like branching and in which a "reflector" layer internal to the actual photogenic tissue is lacking; (3) those like Type 2 except for having a "reflector" tissue; (4) those in which the tracheae run through the reflector layer, branch out in the interface between reflector and photogenic layers, and terminate in "tracheal end-cells" from which minute tracheal capillaries or tracheoles run into the photogenic tissue; (5) those like Type 4 except that the tracheae branch within the photogenic layer before terminating in end-cells and tracheoles; (6) those in which the tracheae run vertically through the photogenic layer in tissue rods called "cylinders", which contain the tracheal twigs and usually end-cells, and from which the tracheoles pass into the photogenic tissue. This sixth type occurs in most of the common adult American fireflies, while the third type is characteristic of firefly larvae.

3. The reflector layer has been shown to differ chemically and morphologically from the photogenic layer. No clear-cut evidence has been adduced as to the function or functions of the reflector layer, although numerous writers have postulated metabolic connections between it and the photogenic layer.

4. The location and morphology of the tracheal end-cell, and the fact that it is the chief site of reduction of inspired osmium tetroxide vapor, have engendered many suggestions that it functions in controlling the oxygen used in luminescence. However, tracheal end-cells, of which there appear to be at least two types, show no conclusive morphological evidence of being able to function in the way postulated.

5. Microscopic observation has shown that the light originates in the photogenic cells. The granules which are a characteristic feature of these cells are almost universally regarded as being the source of light, but no morphological evidence exists which confirms this assumption, nor the view, held by some investigators, that the granules are symbiotic luminous bacteria.

6. The anatomical course of firefly tracheoles strongly suggests that they function in conducting air to the photogenic tissue, and there is strong inferential evidence of the normal presence of air in the tracheoles, although this has not been demonstrated directly. Great variation exists in different organs, but in the most complex type the majority of evidence indicates that the tracheoles pursue an intercellular, rather than intracellular, course and anastomose directly with tracheoles from adjacent cylinders.

7. Nerves to photogenic cells or end-cells, or both, have been described. Experimental evidence indicates strongly that the nervous system, while not necessary for the production of light, does play an essential role in the control of luminescence in the normal living firefly. No conclusion can yet be drawn as to whether the nervous control is exerted directly upon the photogenic tissue, or secondarily (by way of the oxygen supply, for example). However, there is considerable evidence suggestive of the former.

8. By silver nitrate impregnation, a structure can be demonstrated which resembles an ultra-tracheolar network of tubules connecting neighboring tracheoles.

9. The normal types of light-emission in various fireflies fall into four increasingly complex classes: (a) the continuous glow; (b) the intermittent glow; (c) the pulsation; (d) the flash (of which there are several types). If various kinds of fireflies are arranged in order of increasing complexity of the air-supply to the photogenic organ, that order corresponds well to the above sequence of complexity of light-emission.

10. In the more complex types of firefly luminous organ, there are many thousands of photogenic units, each of which is active for a few hundredths or perhaps thousandths of a second.

11. Several hypothetical mechanisms for intracellular control of luminescence have been proposed.

12. It is probable that neither water, luciferin, nor luciferase is a limited reactant in the normal control of luminescence in the firefly.

13. It is certain that oxygen is essential for continued luminescence, and possible that it is the regulated reactant in the control of light-production in the firefly.

14. The spiracles have no causal connection with the control of normal flashing, or of glows caused by various experimental treatments. It is likely, however, that spiracular opening is prerequisite to long-continued luminescence.

15. The "internal control" of luminescence responds in a graded, rather than in an all-or-none fashion, and over-compensates in "opening" in response to lowered oxygen.

16. The simplest assumption in regard to the normal states of both spiracles and internal control is that they are in tonic contraction (closed). Partial anoxia, anesthesia and similar treatments would, thus, produce a relaxed (open) condition, permitting luminescence.

17. There is no anatomical evidence specifically identifying the internal control with the tracheal end-cell, although in the more complex types of firefly organ, where luminescence can be controlled rapidly, there is strong indirect evidence that the tracheal end-cell is concerned in control. However, firefly larvae, which lack end-cells, exhibit internal control of glowing similar to that in adults, indicating that end-cells need not be concerned with glow control even if they are concerned in flash control.

18. Some theoretical implications of the hypothesis that light-production is controlled by competition for oxygen between the luminescent reaction and respiration are discussed.

19. The hypothesis that access of oxygen to the luminescent tissue is controlled by changes in the fluid content of the tracheoles, is discussed and found to be deficient in some respects.

20. The hypothesis that oxygen access is limited by a valvular action of the tracheal end-cells is discussed in detail and found to accord well with what is actually known at the moment about control of flashing in the firefly. Like others, however, the end-cell hypothesis requires the use of a number of assumptions which, so far, it has been impossible to test experimentally.

21. Arguments are presented for regarding enzymatic mechanisms as better fitted than oxygen regulation for the rapid and complete control of luminescence.

Almost all of the work reported in this paper was concerned, either directly or indirectly, with the general question of what mechanisms, external to the photogenic cell, affect its luminescence. In considering the possible trends of future work in this field, it seems clear that important new departures cannot be expected until definite solutions are obtained to a number of long-recognized basic problems. Most of these problems center around the question of whether the photogenic cell is stimulated by direct nerve action, presumably in an environment always adequate in oxygen, or whether the stimulus which sets off luminescence is the passage of oxygen into the cell.

As already related, it has so far been impossible to devise a physiological experiment by which the respective effects of nerve action and of oxygen can be separated with certainty. One of the most promising future experiments is the simultaneous recording of light-emission and of the spontaneous action potentials of the central nervous system. Yet, even if a good correlation were found between the potential pattern and luminosity, direct nervous action would not be proved. It could be argued that the nerves had been stimulating some indirect agency, such as the end-cells, which, in turn, brought about direct stimulation of the photogenic cell by oxygen. Another significant type of approach involves a careful physiological study of two species of lampyrid fireflies, one of which has end-cells and the other has not.

The most direct lines of attack, at the moment, seem anatomical. Conclusive establishment of the ultimate terminations of the nerves to the photogenic organ, if carried out both in fireflies with end-cells and in those without, might go far in resolving the present impasse. Conversely, any cytological evidence bearing on the structure and activity of the end-cells and tracheoles in the living photogenic organ, both in the luminescent and non-luminescent state, could hardly fail to be significant. Likewise, evidence that end-cells can regulate oxygen rapidly in non-luminous insects, would be of great value. Furthermore, it would be of at least theoretical interest to calculate the potentialities of gaseous diffusion, flow, and pressure, in supplying air to the photogenic tissue through conducting systems of the known anatomical dimensions.

Further important information on the possibility of direct nerve action might be obtained by carefully controlled experiments on electrical stimulation of the nerve cord, with special reference to the relation between length or strength of stimulus and total resulting luminescence, total luminescence in relation to oxygen tension, and total luminescence in relation to length of preceding anaerobiosis. Oxygen tensions used should be kept within the range of 100 to 300 mm., in which many physiological reactions remain constant, and particular attention should be paid to fireflies lacking end-cells.

Finally, the simplest and most important desideratum in future work on fireflies is improvement in the design of experiment. It cannot fail to impress anyone familiar with the literature, that an enormous amount of work has been weakened or negated by failure to observe such elementary methodological precautions as the use of adequate controls, frequent repetitions, the variation of only one experimental factor at a time, and appreciation of the range and frequency of the normal and experimental variation of the material.

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* No attempt has been made to present a complete bibliography on fireflies, but I have tried to include all the important contributions, particularly those which have appeared since Dahlgren's review. Wherever possible, only a single paper (the most comprehensive, rather than the first) is cited for a given author. No references are included which are not mentioned in the text. References marked with an asterisk were not available in the original, and are quoted from the reviews of **Edwards** (1863), **Kerville** (1881, 1887), **Henneguy** (1904), **Höllrigl** (1908), **Berlese** (1909), **Mangold** (1910), **Kastle & McDermott** (1910), **Harvey** (1920, 1940, and 1941), and **Pratje** (1923). I am indebted to Professor Harvey for the opportunity to read the papers of Hasama, which were not otherwise available in this country.

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FIGURES 1-41

Key

C, cylinder; CM, cell membrane; CU, cuticle; DZ, differentiated zone of photogenic cytoplasm; EC, tracheal end-cell; EN, end-cell nucleus; EP, end-cell process; FB, fat body; M, muscle; N, nerve; NE, epithelial cell nucleus; O, region where osmium has reduced in the photogenic cytoplasm; P, photogenic layer or tissue; PC, photogenic cell; PN, nucleus of photogenic cell; R, reflector layer; RC, reflector cell; S, "rounded body"—a possible sphincter; T, trachea; TE, tracheole; TW, tracheal twig.

Unless otherwise specified in the figure legends, the preparations used for the photomicrographs are 10-micron sections, stained with Delafield's hematoxylin and eosin. Fixation was with Bouin's fluid, except for the preparation for FIGURE 32, for which hot Bouin's was used. Detailed techniques will be published elsewhere.

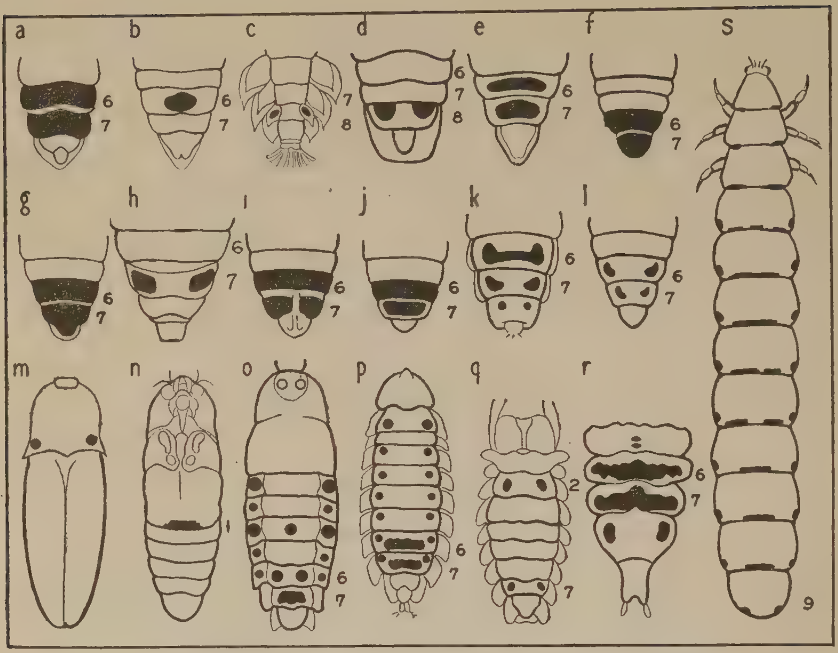


FIGURE 1. Outline diagrams showing positions and sizes of photogenic organs in representative fireflies. Photogenic organs indicated in solid black. Numbers refer to abdominal segments. Ventral views, unless otherwise noted. Sizes are variable and not shown to scale, but normal size of *Phengodes* (s) is about 40 mm., *Pyrophorus* 25 mm., entire "average" firefly 10-15 mm. a, *Photinus scintillans*, male; b, *Photinus scintillans*, female; c, *Photuris pennsylvanica*, larva; d, *Diphotus montanus*, male; e, *Photuris pennsylvanica*, female; f, *Luciola chinensis*, male; g, *Luciola cruciata*, male; h, *Luciola lusitanica*, female; i, *Luciola* sp., male; j, *Luciola lateralis*, male; k, *Pyrocoelia rufa*, female; l, *Lecontea lucifera*, female; m, *Pyrophorus noctilucus*, dorsal; n, *Pyrophorus noctilucus*, ventral; o, *Lamprorhiza* (*Lampyris*) *splendidula*, female (the number of lateral organs is quite variable, according to Vogel, 1922); p, *Phausis mulsanti*, female (the number of lateral organs is variable); q, *Phausis Delarouzeei*, nymph, dorsal; r, *Lampyris noctiluca*, female; s, *Phengodes* sp., dorsal. Figures a, b, c, and e after **Hess** (1920). Figures f, g, i, j, k, and l, after **Okada** (1935 b). Figures b, h, p, and q, after **Bugnion** (1929). Figure o, from various sources. Figure r, after **Bongardt** (1903). Figures d and s, original. Figures m and n from **Seaman** (1891).

FIGURES 2-7 (*see opposite page*).

FIGURE 2. Two end-cells from male of *Lamprorhiza splendidula*. Osmic acid vapor impregnation; x 650. After **Bongardt** (1903).

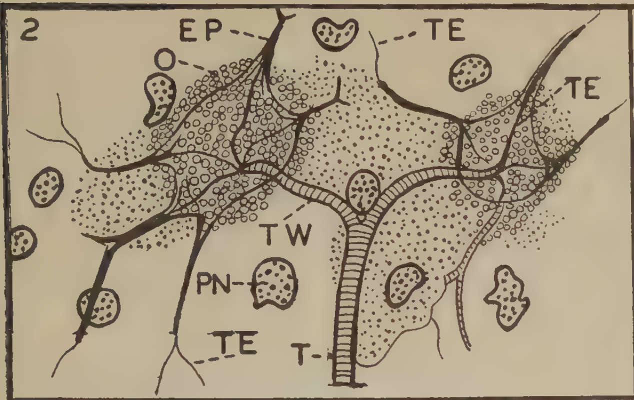
FIGURE 3. Tracheal end-cell from *Photinus marginellatus*. Osmic acid impregnation; x 1700. After **Geipel** (1915).

FIGURE 4. Diagram of Type 4 arrangement of tracheae. End-cells at boundary between reflector and photogenic layers, with tracheoles extending into latter. After **Dahlgren** (1917).

FIGURE 5. Diagram of Type 5 arrangement of tracheae. Trachea branching into interior of photogenic layer and there terminating in end-cells. After **Dahlgren** (1917).

FIGURE 6. Arrangement of end-cells and nerves in the light organ of *Photinus marginellatus*. After **Geipel** (1915).

FIGURE 7. Cross section of the light organ of adult *Photuris pennsylvanica*. After **Hess** (1922). Contrast with FIGURE 11.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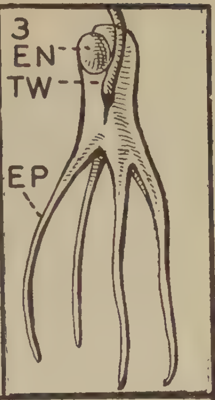
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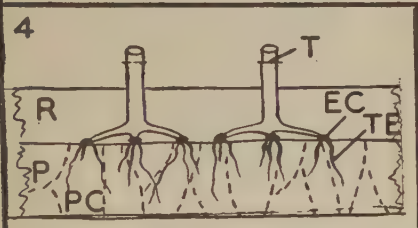
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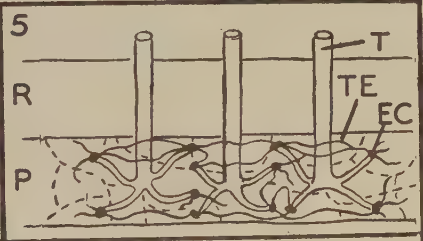
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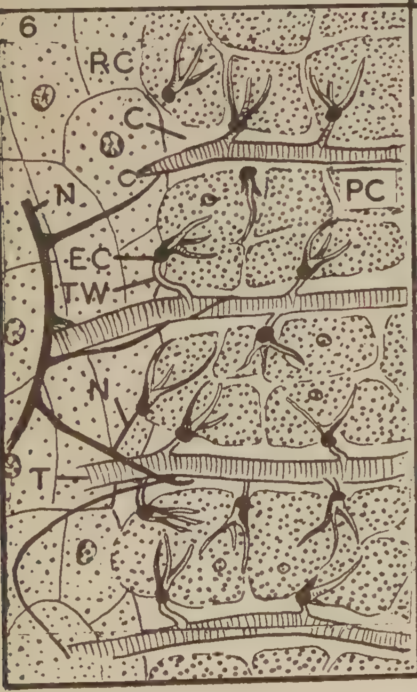
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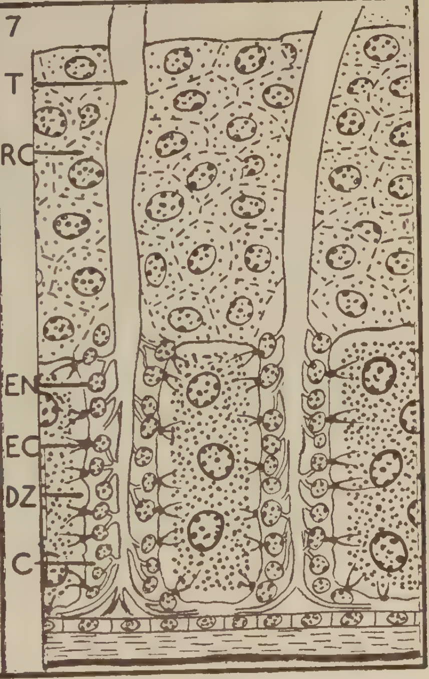
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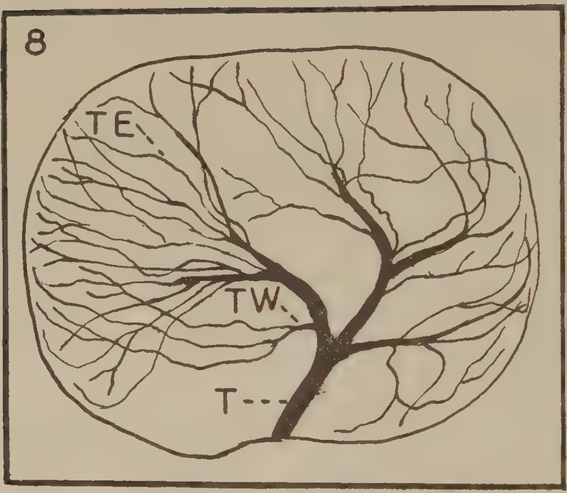


FIGURE 8. Larval organ of *Phosphaenus hemipterus* from caustic potash preparation to show tree-like branching of tracheae. After **Bongardt** (1903).

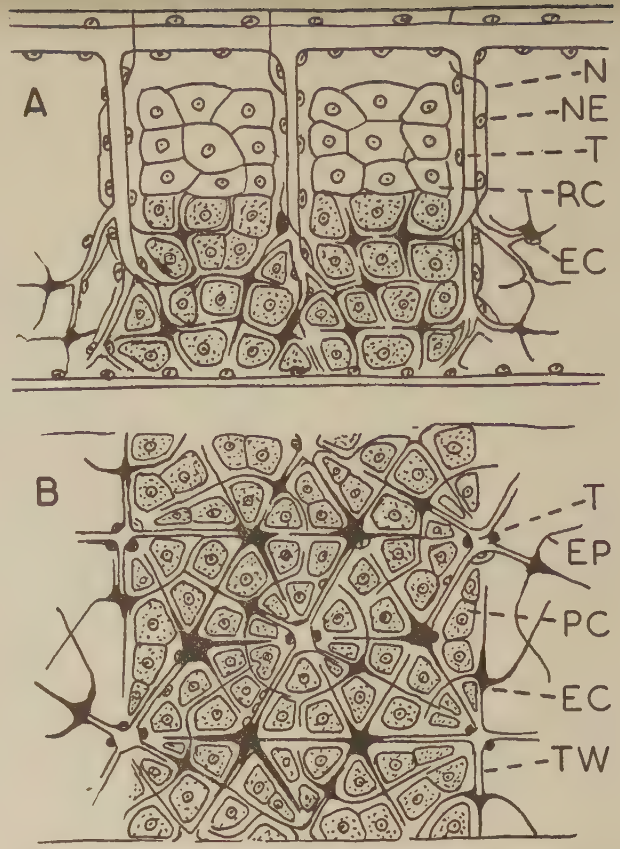


FIGURE 9. Diagram of cross (above) and horizontal (below) sections of light organ of *Luciola cruciata*. This is a variant of the Type 5 system of tracheal branching. After **Okada** (1935 b).

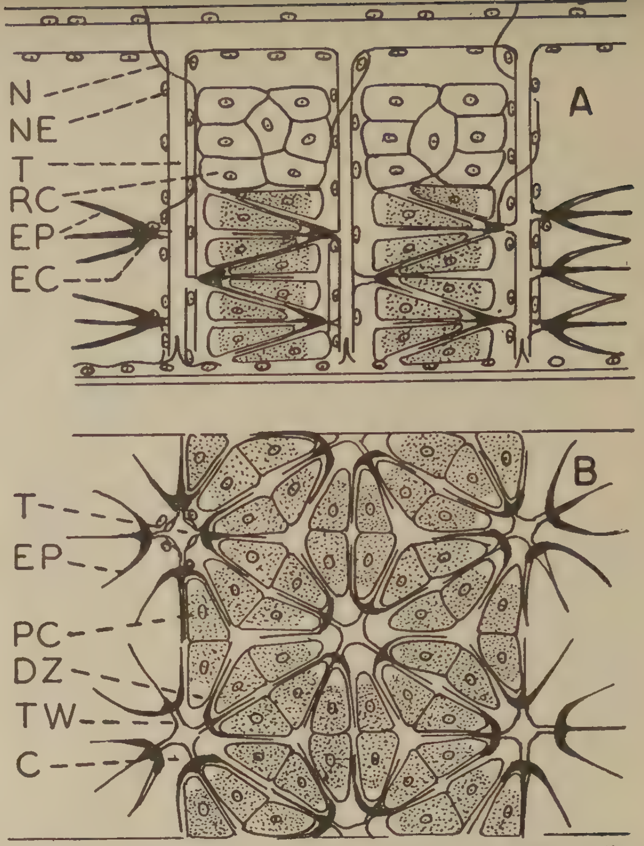


FIGURE 10. Diagram of cross (above) and horizontal (below) sections of the light organ of *Photinus* sp. (Presumably Korean). This is a variant of the Type 6 system of tracheal branching. After **Okada** (1935 b).

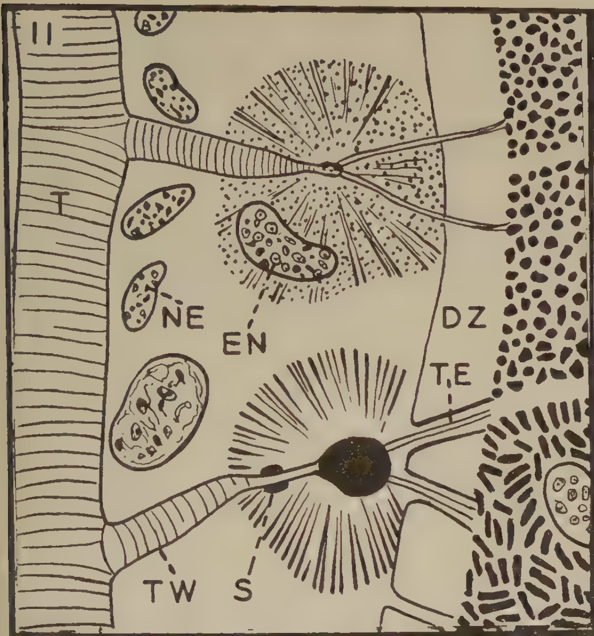
FIGURES 11-14 (*see opposite page*).

FIGURE 11. Composite diagram of end-cell and cytoplasmic structure from Figures 11, 16 and 20 of **Dahlgren** (1917). Lower end-cell more heavily impregnated with osmic acid than upper, and showing "fibers" in its cytoplasm and a "rounded body" (s) with postulated constrictor powers. Rounded photogenic granules in upper cells of male type; rod-shaped granules in lower photogenic cell of female type.

FIGURE 12. Cross section of light organ of *Pyrophorus*. After **Dubois**.

FIGURE 13. Diagram of horizontal section through light organ of *Photinus marginellus* to show "rosette" pattern of tracheolar anastomoses between cylinders. Diameters of tracheoles exaggerated. Upper two cylinders show terminal brushes of tracheal twigs, lower shows nuclei of tracheal epithelium and end-cells; approx. x 1000. Partly after two figures of **Townsend** (1904).

FIGURE 14. Cross section of light organ of female of *Photuris pennsylvanica* from osmic acid preparation. After **Lund** (1911).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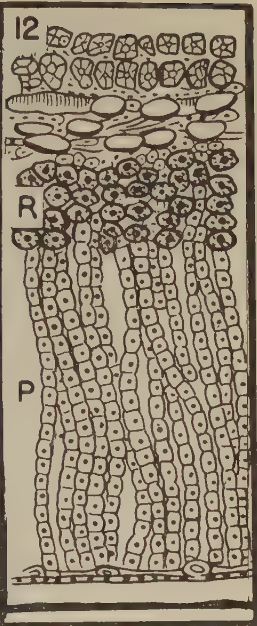
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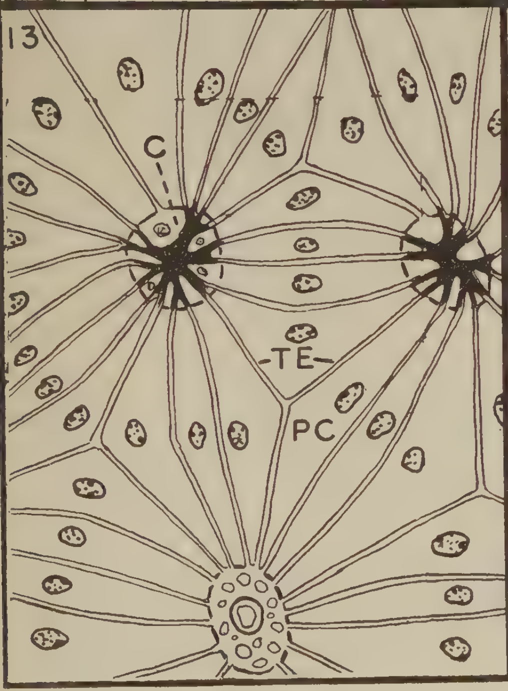
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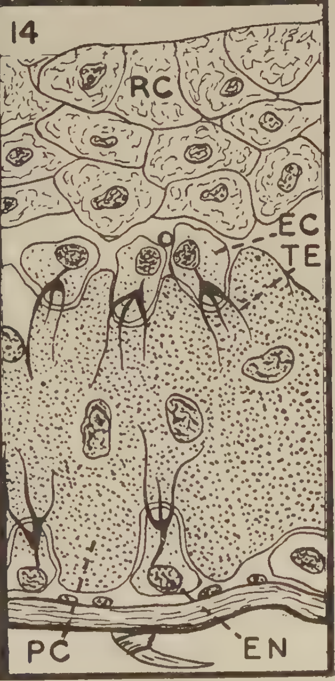
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PLATE 1

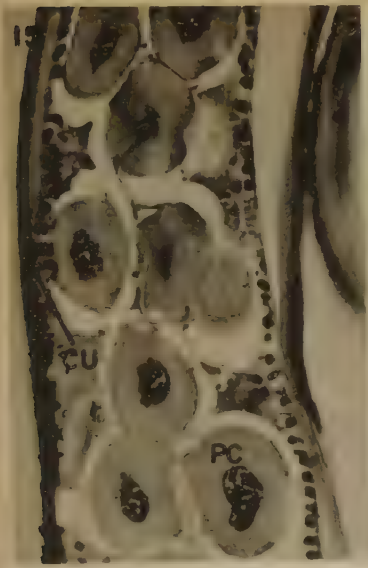
FIGURE 15. *Phengodes* sp. Longitudinal section of lateral luminous organ; X180. Large oenocyte-like photogenic cells loosely aggregated in a fold of the external cuticle. The photogenic cells are apparently slightly shrunken.

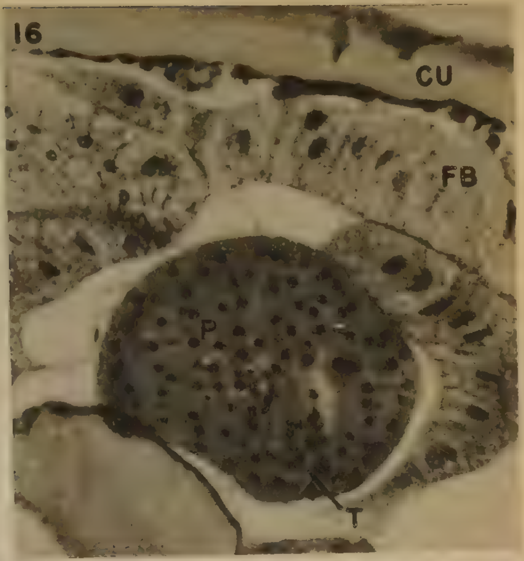
FIGURE 16. *Phrixothrix* sp. Cross section of lateral luminous organ; X200. Organ is situated amid fat-bodies beneath the dorsolateral cuticle. Clear spaces between photogenic cells are small tracheal branches.

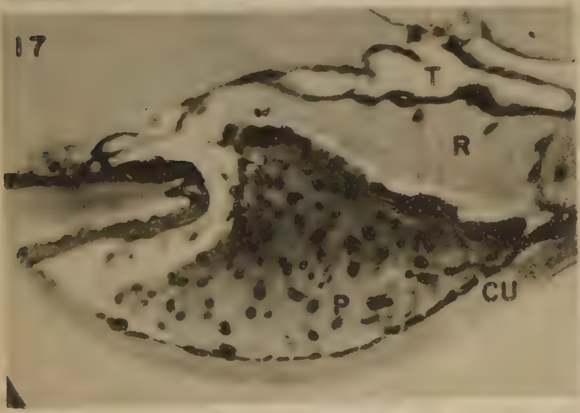
FIGURE 17. *Photuris pennsylvanica.* Cross section of luminous organ of larva; X180. The two white dots at the apex of the photogenic layer are cross-sections of tracheae. Ventral cuticle slightly retouched.

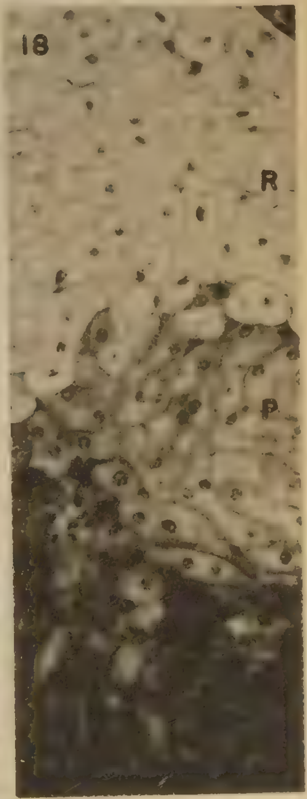
FIGURE 18. *Pyrophorus plagiophthalmus.* Cross section of abdominal organ; X250.

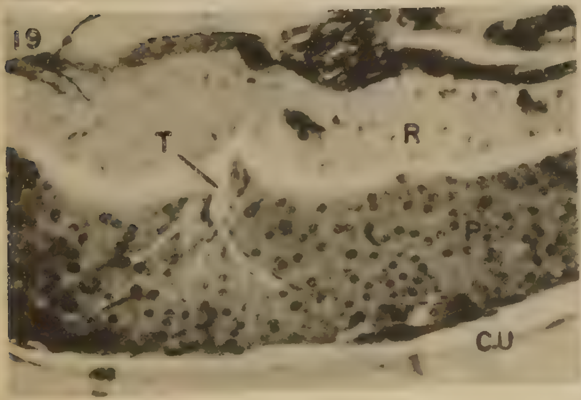
FIGURE 19. *Diphotus montanus,* female. Cross section of part of luminous organ; X250. The separation between photogenic and reflector layers is an artifact.



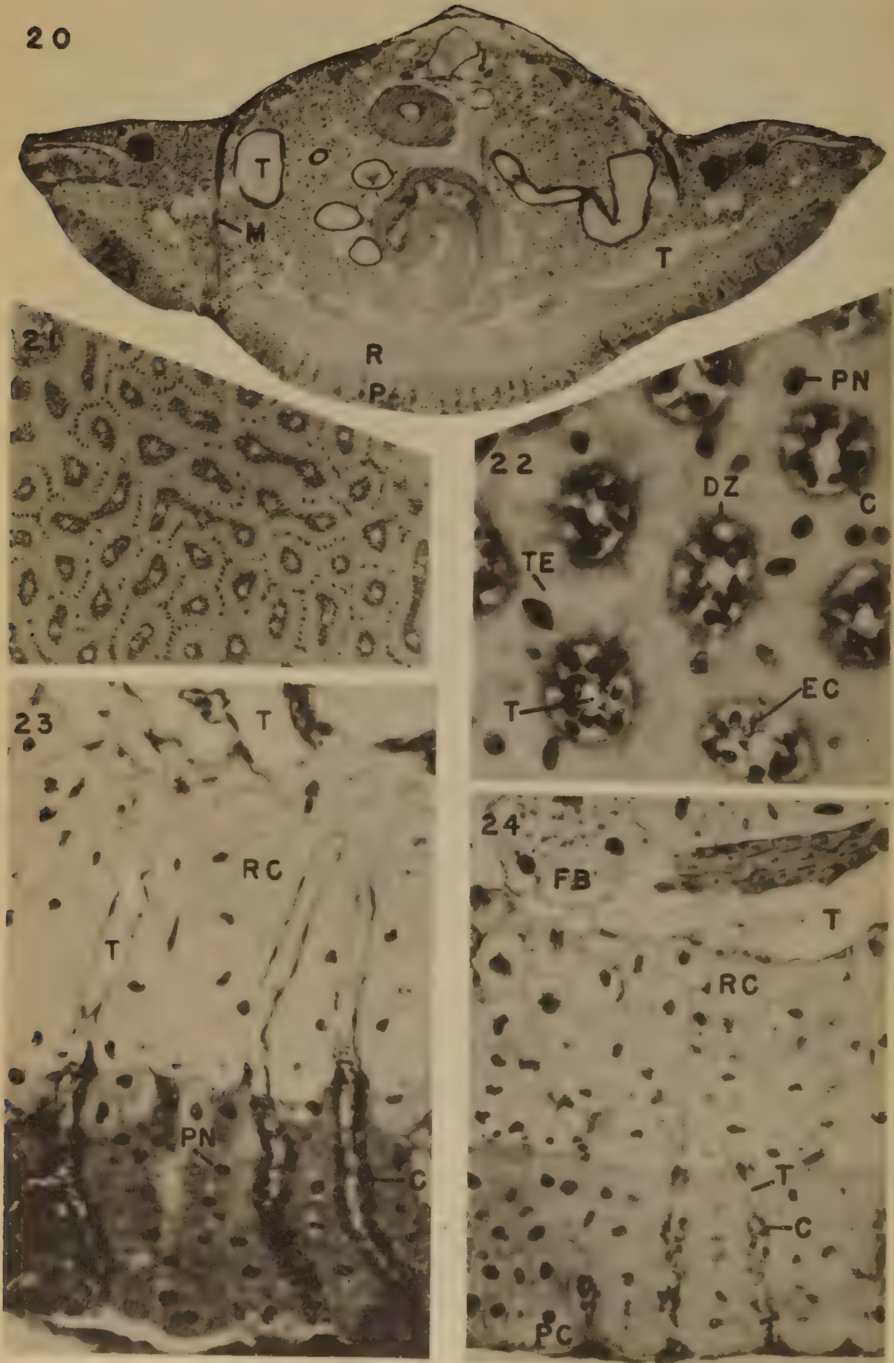








BUCK: THE LIGHT ORGAN IN FIREFLIES



BUCK: THE LIGHT ORGAN IN FIREFLIES

PLATE 2

FIGURE 20. *Photuris jamaicensis,* female. Cross section through entire abdomen, to show location of light organ on the ventral surface; X25. Note the high concentration of large tracheae on the internal surface of the reflector layer.

FIGURE 21. *Photinus xanthophotis catherinae,* male. Horizontal section through photogenic organ, to show the tracheal trunks arranged in triangular symmetry and the "rosettes" of photogenic cells around each; X96. The nuclei of the photogenic cells are arranged approximately equidistant from adjacent cylinders. The nuclei grouped close around each trachea belong mainly to tracheal end-cells, which are particularly abundant at the ventral surface of the organ (see also FIGURE 39). Compare with FIGURES 22, 29, and 36.

FIGURE 22. *Photinus euphotus,* male. Horizontal section through photogenic organ, showing "rosette" arrangement of photogenic cells around the cylinders: X540. This view also shows end-cells, tracheoles, cylinders, and the differentiated zone of the photogenic cytoplasm. Compare with FIGURES 21, 29, and 36.

FIGURE 23. *Photinus pyralis,* male. Cross-section through light organ; X250. This organ is characterized by large tracheal trunks, long narrow cylinders, photogenic and reflector layers of approximately the same thickness and number of cell layers (7 or more). One or two of the most internal of the photogenic cells show a lighter cytoplasm, such as has been described for "transition cells" between the photogenic and reflector layers (page 406). Compare with FIGURES 24, 25, 26, 31, 32, and 35.

FIGURE 24. *Photinus synchronans,* male. Cross-section through light organ: X250. This organ has narrow tracheae, and photogenic and reflector layers each about 5 cells thick. Compare with FIGURES 23, 25, 26, 31, 32, and 35.

PLATE 3

FIGURE 25. *Photuris jamaicensis,* male. Cross-section through luminous organ; X250. In this species, the photogenic layer is usually thinner than the reflector, and the cylinders are often greatly flared at the ventral (and often the dorsal) surface of the photogenic layer. Compare with FIGURES 23, 24, 26, 31, 35, and especially 32.

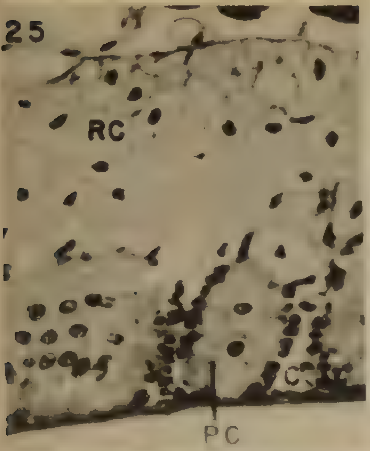
FIGURE 26. *Photinus pallens,* male. Cross-section through photogenic organ; X230. Specially stained (first method of Severinghaus) to accentuate the differentiated zone of the photogenic cells. Also shows cylinders expanded in the interior of the photogenic layer, and tracheoles. Compare with FIGURES 23, 24, 25, 31, 32, and 35.

FIGURE 27. *Photinus pallens,* male. Three separated cylinders from osmic acid maceration preparation; X500. The end-cells show varying degrees of impregnation from light (in the upper part of the right-hand cylinder) to heavy (in the upper part of the left-hand cylinder). A few tracheoles show in upper left and lower right. Note the profusion of tracheal twigs and end-cells.

FIGURE 28. *Photinus pyralis,* male. Ventral surface view of photogenic organ which has been freshly peeled off; X500. The brushes of tracheae and twigs are especially profuse at the surface, since the cylinders usually flare there. Dark "wall" of cylinders may be the differentiated zone of the photogenic cytoplasm. Compare with FIGURES 29 and 38.

FIGURE 29. *Photinus pyralis,* male. Ventral view of photogenic organ which has been peeled off and partly dried; X790. Deeper focal level than FIGURE 28. Note tracheoles running between contiguous cylinders (several anastomoses are visible). Origin of tracheoles within cylinder visible in the central cylinder and the one to the left of it. Compare with FIGURES 28 and 38.

FIGURE 30. *Photinus pallens,* male. Lateral view of terminal part of tracheal trunk of cylinder, from fresh smear; X500.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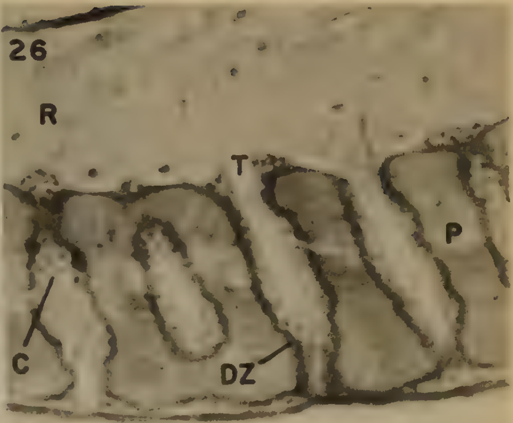
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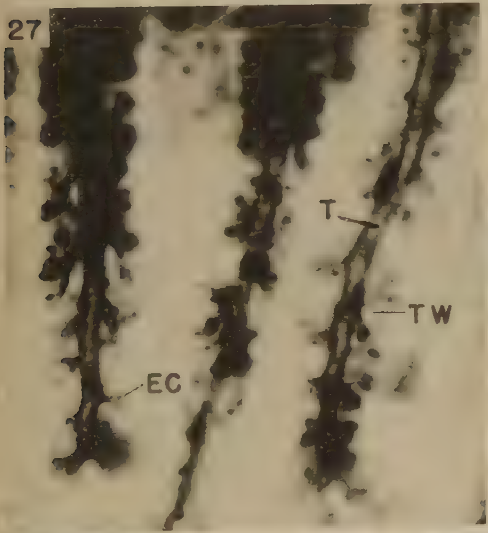
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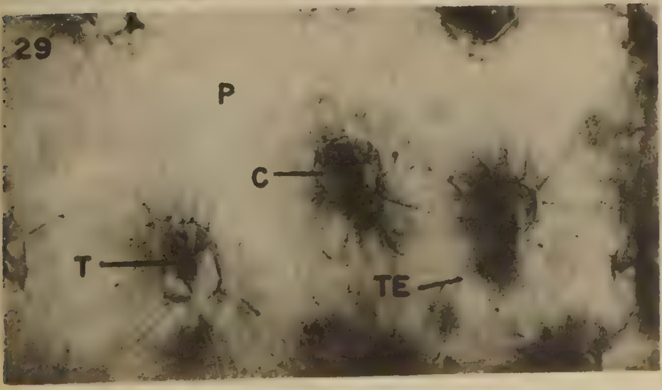
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BUCK: THE LIGHT ORGAN IN FIREFLIES

PLATE 5

FIGURE 35. *Photuris pennsylvanica,* female. Cross-section through photogenic organ; X830. This figure shows cell membranes (running vertically), along which appear cross-sections of tracheoles. Note also that the photogenic layer is only one cell thick and that its cells are very thin. No cylinder shows in this view. Compare with FIGURES 23, 24, 25, 31, and 32.

FIGURE 36. *Photinus pallens,* male. Ventral surface view of silver nitrate smear; X630. The membranes of the photogenic cells are seen running radially from the cylinders in a rosette pattern. Tracheoles viewed end-wise appear as small circles strung along the membranes. Compare with FIGURES 21, 22 and 29.

FIGURE 37. *Photinus pallens,* male. Osmic acid smear, showing isolated cylinder tissue with tracheae and lightly impregnated end-cells and tracheoles; X500. Origin of two tracheoles from one end-cell, and probable anastomoses, are visible.

FIGURE 38. *Photinus pyralis,* male. Ventral view of partly dried photogenic organ, at level closer to surface than that shown in FIGURE 29; X790. This view shows the mode of origin of the tracheoles from the ends of the tracheal twigs within the cylinders. End-cells and other cytological details are invisible, since the preparation is unstained.

FIGURE 39. *Photinus xanthophotis catherinae,* male. Ventral surface view of organ peeled off after impregnation with osmic acid vapor; X425. This view shows the profuse brushes of end-cells which crowd the cylinders at the ventral surface where they flare. Note profuse tracheolar supply with many inter-cylinder anastomoses.

FIGURE 40. *Photuris jamaicensis,* male. Cluster of end-cells from terminal brush in flared ventral region of cylinder, from osmic acid smear; X500.

FIGURE 41. String galvanometer record of single spontaneous flash of *Photuris pennsylvanica.* Vertical axis, light intensity in arbitrary units; horizontal axis, time in 0.04 sec. intervals. From **Snell,** 1932.

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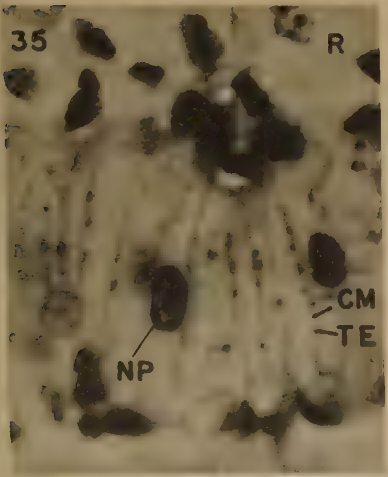

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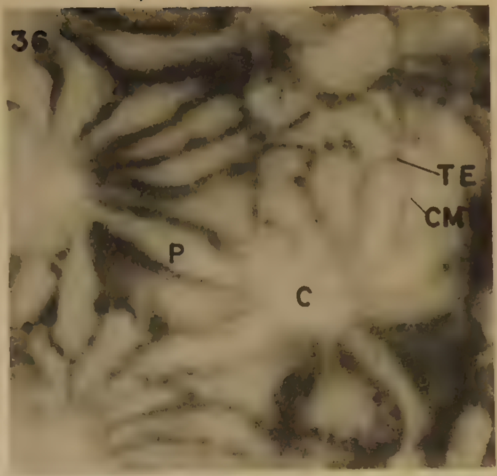

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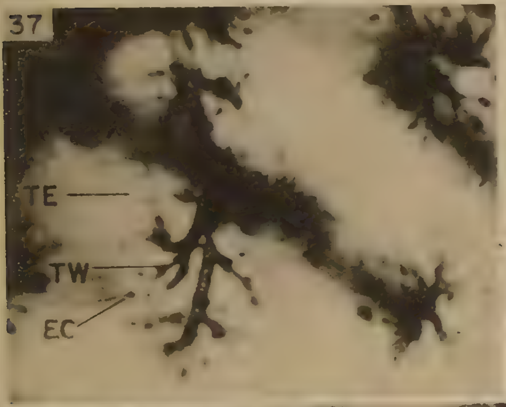

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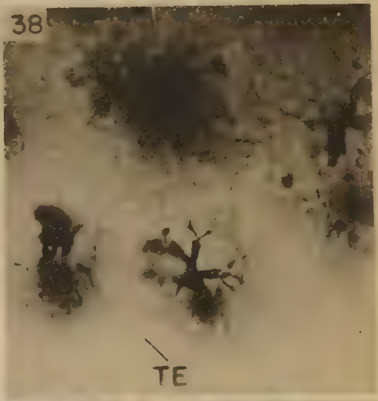

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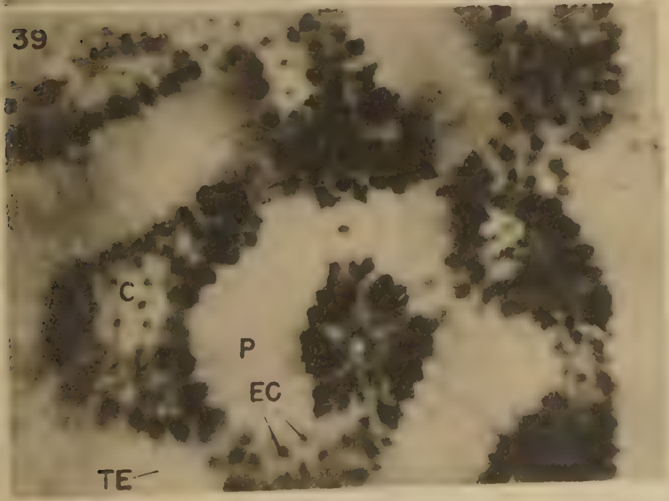

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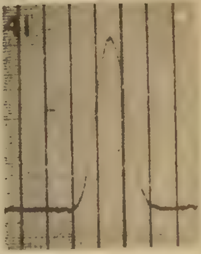

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